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#### (57) Abstract

The present invention relates to primers that specifically hybridize to nucleic acid molecules complementary to the messenger RNA transcribed from genes encoding MAGE tumor-specific antigens or a part thereof or to a complementary strand thereof encoding MAGE tumor-specific antigens as well as to diagnostic compositions comprising said antigens. The present invention further relates to methods for detecting disseminated tumor cells employing the primers of the invention as well as methods for preparing a tumor adjuvant vaccine.

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## NOVEL PRIMERS AND METHODS FOR THE DETECTION OF DISSEMINATED TUMOR CELLS

The present invention relates to primers that specifically hybridize to nucleic acid molecules complementary to the messenger RNA transcribed from genes encoding MAGE tumor-specific antigens or a part thereof or to a complementary strand thereof as well as to diagnostic compositions comprising said antigens. The present invention further relates to methods for detecting disseminated tumor cells employing the primers of the invention as well as methods for preparing a tumor adjuvant vaccine.

Immunotherapeutic approaches have previously been applied to patients with minimal residual disease and could reduce mortality and recurrence associated with distant metastases. Since early occult dissemination of tumor cells is already present in about half of the cancer patients, efficient treatment strategies have to be developed for eliminating residual tumor cells. Many immunocytochemical and PCR-based assays using histogenetic differentiation markers exclusively allow the detection of micrometastatic cells that worsen the prognosis after local tumorectomy without identifying antigens for adjuvant immunotherapy. Limitations of RT-PCR analyses in oncological disorders resulted in the tissue-specific rather than tumor-specific expression of marker genes. Detection of disseminated tumor cells is thus restricted to a few, certain tumor types deriving from similar histological origin. Moreover, tumor cells are only detectable in front of special tissue background, so that most published approaches depend on samples taken from mesenchymal compartments. The presence of ubiquitous transcriptional factors and minimal activation of promotor sequences in background cells may cause illegitimate transcription and reduce specificity and sensitivity due to

competing background signals. Furthermore, most assays measure only a single marker gene not considering inter- and intra-individual tumor cell heterogenity.

Several tumor rejection antigens have recently been characterized that are presented on major histocompatibility complex (MHC) class I molecules to autologous cytolytic T-lymphocytes (CTL). The MAGE genes belong to a family of 12 closely related genes with an overall homology of 64-85% and are classified as "cancer-testis-antigens".

The MAGE genes have been employed in the prior art to use primers specific for MAGE 1 to 4, 6 and 12 for the detection of a variety of tumors (WO 95/23874). The specific primers as well as the regions they prime to in the MAGE genes are, however, inappropriate for the diagnosis of disseminated tumor cells in human samples. WO 96/29430 discloses genetic markers for the detection of melanomas and breast cancer cells including occult cancer cells or metastatic cells derived therefrom. In said application, it is further described that a number of markers including MAGE-3 may be employed to detect such tumors. However, methods disclosed in this application are not expected to be suited for the detection of discerninated tumor cells derived from human malignacies of many different histological origins.

Since, in particular, the early reliable detection of disseminated fumor cells in a patient enables the early treatment and/or vaccination in order to prevent recurrence of cancerous growth, the technical problem underlying the present invention was to improve the sensitivity of the prior art methods for detecting such disseminated tumor cells in patients with maligancies of many different histological origins. The solution to said problem is achieved by the embodiments characterized in the claims.

Accordingly, the present invention relates to a primer specifically hybridizing to a nucleic acid molecule complementary to the messenger RNA transcribed from a

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gene encoding a MAGE tumor-specific antigen or a part thereof or to a complementary strand thereof, said primer being selected from the group of

(i) primers having the same 3' portion as one of the following groups of primers:

(a)

5'-gtagagttcggccgaaggaac-3'	(SEQ ID NO:1
5'-caggagctgggcaatgaagac-3'	(SEQ ID NO:2)
5'-cattgaaggagaagatctgcct-3'	(SEQ ID NO: 3)
5'-gagtagaagaggaagaagcggt-3'	(SEQ ID NO: 4)
5'-gaageeggeecaggeteg-3'	(SEQ ID NO: 5)
5'-gatgactetggteagggeaa-3'	(SEQ ID NO: 6)
5'-caccaaggagaagatctgcct-3'	(SEQ ID NO: 7)
5'-tcctcagtagtaggagcctgt-3'	(SEQ ID NO: 8)
5'-tccgtgaggaggcaaggttc-3'	(SEQ ID NO: 9)
5'-atcggattgactccagagagta-3'	(SEQ ID NO: 10)
(b)	
5'-tagagttcggccgaaggaac-3'	(SEQ ID NO: 11)
5'-ctgggcaatgaagacccaca-3'	(SEQ ID NO: 12)
5'-cattgaaggagaagatctgcct-3'	(SEQ ID NO: 13)
5'-caggcttgcagtgctgactc-3'	(SEQ ID NO: 14)
5'-ggctcggtgaggaggcaag-3'	(SEQ ID NO: 15)
5'-gatgactctggtcagggcaa-3'	(SEQ ID NO: 16)
5'-caccaaggagaagatctgcct-3'	(SEQ ID NO: 17)
5'-caggcttgcagtgctgactct-3'	(SEQ ID NQ: 18)
5'-tccgtgaggaggcaaggttc-3'	(SEQ ID NO: 19)
5'-gagcctgcgcacccaccaa-3'	(SEQ ID NO: 20)
	•

and;

(ii) primers that overlap with a primer sequence depicted in (a) or (b).

The term "overlap" in this context means the overlap of at least one nucleotide.

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Accordingly, said primers can hybridize to RNA, preferably mRNA or to DNA, preferably cDNA.

In accordance with the present invention, it was found that the primers having the above-recited 3' ends are particularly useful in detecting disseminated tumor cells of many different histological origins at a sensitivity level that was unknown from the prior art. Additionally, primers that overlap with the above-recited nucleic acid sequences and that hybridize to MAGE genes have been found useful for the detection of disseminated tumor cells in human samples. Preferably, said primers overlap with the nucleic acid sequences depicted under (a), above. It is further preferred that the primers of the invention have a length of between 15 and 25 nucleotides. With the exception of primers that recognize MAGE-3 and -6, each of asid primers specifically detects the expression of only one MAGE gene.

occurs under stringent conditions. Such conditions can b d vised by the person skilled in the art according to conventional protocols. The specific hybridization antigens in a sensitive RT-PCR. Such RT-PCRs can be devised by the person not cross-amplify nucleic acids from the genes of other MAGE tumor specific accordance with the present invention is intended to mean that these primers do enough for such an analysis. The term "specifically hybridizing" as used in primers has, in accordance with the present invention, proven not to be high malignancies of many different histological origins since the sensitivity of said suitable for detecting disseminated tumor cells in samples of patients with the MAGE genes to which the primers described in the prior art hybridize are not be emphasized that the prior at methods and primers as well as the regions of primers, even if the analyzed patient has no clinical evidence of disease. It has to cells of many different histological origins can be detected using the above-recited by the present invention that, in contrast to prior art reports, disseminated tumor of patients where disseminated tumor cells are detected. It was surprisingly found condition at a very early stage of the disease and thus allows the timely treatment The present invention accordingly provides a means for detecting a cancerous

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skilled in the art without undue burden according to conventional protocols; see, e.g., "Nucleic Acid Hybridization, A Practical Approach"; edited by Hames & Higgins, IRL Press, Oxford 1985.

The primers of the invention are advantageously used in PCR amplification of nucleic acid sequences derived from the desired MAGE genes or fragments thereof. The primers of the invention are devised to avoid the amplification of genomic DNA but to amplify a DNA sequence derived from the reverse transcription of messenger RNA. In this way, the PCR product can be unambiguously correlated with expressed MAGE-genes. PCR may be carried out according to conventional methods. Also, the PCR product may be detected according to protocols that are established in the art.

Preferably, the primer of the invention is a primer depicted in either group (a) or group (b), supra.

The primers of this embodiment have been particularly useful and advantageous in identifying disseminated tumor cells in samples of patients with malignancies of many different histological origins. It is also particularly preferred that primers of group (a) are used as primers for a first round of PCR amplification whereas primers of group (b) are used in a nested primer PCR amplification.

The present invention further relates to a diagnostic composition comprising at least four primers according to the invention wherein said at least four primers hybridize pair-wise to strands of opposite orientation of at least two different nucleic acid molecules wherein strands of one orientation are complementary to the messenger RNA transcribed from the genes of at least two different MAGE tumor-specific antigens.

The diagnostic composition of the invention is particularly useful for carrying out a variety of PCRs and thus for unambiguously identifying the presence of disseminated tumor cells in patients with malignancies of many different histological origins. As has been found in accordance with the present invention,

at least two different MAGE-genes from the group of MAGE-1,-2,-3,-4, -6 and -12 should be checked for expression in order to further secure the diagnostic value of the investigation. Preferably, said diagnostic kit comprises at least one pair of primers specific for each of the MAGE-1,-2,-3,-4, -6 and -12 genes. It is further preferred that the analysis of the sample is carried out with the kit of the invention until the expression of at least two different MAGE-genes has been secured.

The bottling of the primers in said kit is effected according to conventional procedures. Preferably, each primer is separately bottled. Alternatively, the primers specific for each MAGE-gene are bottled together with the understanding that primers for a primary and a nested primer reaction are separately bottled.

The present invention further relates to a method of detecting disseminated tumor

cells indicative of a cancerous condition in a patient comprising:

(a) carrying out PCR on cDNA obtained from mRNA from one or more patient

carrying out PCR on cDNA obtained from mRNA from one or more patient samples using at least two primers of the invention hybridizing pair-wise to strands of opposite orientation of at least two one cDNA molecule wherein strands of one orientation are complementary to the messenger RNA strands of one orientation are complementary to the messenger RNA transcribed from the genes of at least one MAGE tumor-specific antigen; and

(b) detecting one or more PCR products.

The term "cancerous condition" is intended to mean the whole spectrum from the initiation of malignant transformation in single cells to advanced cancer disease

including distant solid metastasis.

Preferably, at least four primers specific for at least two MAGE transcripts and complementary strands thereof are employed in the method of the invention.

Additionally, the present invention relates to a method of preparing a tumor adjuvant vaccine comprising the following steps:

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(a) carrying out PCR on cDNA obtained from mRNA from one or more patient samples using at least four primers of the invention hybridizing pair-wise to strands of opposite orientation of at least two different nucleic acid molecules wherein strands of one orientation are complementary to the messenger RNA transcribed from the genes of at least two different MAGE tumor-specific antigens;

- (b) detecting resulting PCR products; and
- (c) using at least one MAGE-gene product, expression of said MAGE-gene being detected as a result of step (b) for preparing an adjuvent tumor vaccine based on MAGE-antigen-derived peptides resembling MHCrestricted T-cell-epitopes, whole MAGE-antigens or fragments thereof, MAGE-transfected host cells, DNA vaccinating strategies using MAGEencoding nucleotide sequences or any other immunization method based on MAGE-gene products or their coding nucleotide sequences.

This embodiment of the invention relates to the direct application of the positive analysis of the presence of disseminated tumor cells in a human sample. Once said tumor cells have been detected, a suitable antidote for the further proliferation of said tumor cells eventually resulting in tumorous growth should be devised. The method of the present invention provides such an antidote. The tumor adjuvant vaccine can be prepared according to conventional protocols. In particular, it is envisaged to pulse dendritic cells isolated from the patient with peptides derived from the coding sequences of those MAGE-genes detected to be expressed by the disseminated tumor cells of this particular patient using the diagnostic composition of the present invention, these peptides being suited for binding to MHC-molecules of this patient thus resembling MHC-restricted T-cells epitopes. The peptide-pulsed dendritic cells will then be reinjected into the patient and will migrate to the T-cell zones of secondary lymphoid organs where they prime MAGE-specific T-lymphocytes thus resulting in an immune response against MAGE-positive tumor cells.

In a preferred embodiment of the method of the invention, said cDNA is obtained by:

- (a) preparing mRNA from one or more samples removed from said patient;
- (b) reverse transcribing said mRNA and the reverse transcription thereof

The preparation of said mRNA and the reverse transcription thereof is conveniently effected by conventional procedures.

Reverse transcription of mRNA resulting in the corresponding cDNA may be carried out by random hexanucleotide priming as described in Example 3 by oligo-dT-priming or by specific priming using one or more oligonucleotides complementary to oneor more MAGE-mRNA species. Due to the high degree of sequence homology among different members of the Mage gene family, specific primers for the cDNA-synthesis may be identified that efficiently hybridize to the mRNA of more than one Mage gene and most preferably to all the Mage genes mRNA of more than one Mage gene and rested according to the present invention (Mage-1, -2, -3/6, -4 and -12). Such used according to the present invention (Mage-1, -2, -3/6, -4 and -12). Such "pan-Mage primers" can be identified and tested according to the state of the art manuals e.g. Sambrook (Molecular Cloning; A Laboratory Manual, 2<sup>nd</sup> Edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY (1989)) or Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY (1989)) or Hames & Higgins (Nucleic acid hybridization; A practical approach, IRL Press, OxfordWashington DC (1985)).

In an additional preferred embodiment of the method of the invention, said PCR is

nested PCR.

As has been found in accordance with the present invention, the nested PCR is a further safeguard for obtaining unambiguous results with regard to the patient's disease status.

In a further preferred embodiment of the method of the inv ntion, said primers hybridize to 2 to 6 different nucleic acid molecules complementary to the

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messenger RNA transcribed from the genes of MAGE tumor-specific antigens and to complementary strands thereof.

In a further preferred embodiment of the method of the invention, said MAGE tumor-specific antigens are the MAGE-1, -2, 3, -4, -6 or -12 tumor specific antigens.

Of all MAGE antigens, the above-recited antigens most often appear on tumor cells, as was found in accordance with the present invention, and are therefore particularly indicative of the risk of cancer to develop metastatic tumors.

It is further preferred in accordance with the present invention to remove samples from two different aspiration sites. As was found in accordance with the present invention, the analysis of double-sided aspiration significantly improves sensitivity. It is therefore advisable to use double-sides aspiration in the routine diagnosis of tumor cell dissemination.

In a further preferred embodiment of the method of the invention, said samples to be analyzed are bone marrow aspirates.

As has been surprisingly found in accordance with the present invention, the use of bone marrow aspirates as a source for analyzing the metastatic potential of a patient is more sensitive than using peripheral blood as suggested by the prior art.

Additionally preferred is a method wherein said mRNA preparation comprises the following steps:

- (a) immediately lysing the sample in buffer essentially completely avoiding RNA-degradation; and
- (b) optionally centrifuging the mRNA obtained in the lysate through a cushion of an RNAse-inhibiting agent, preferably cesium trifluoracetate.

This embodiment of the invention is advantageous since it precludes the loss of mRNA that is, as is well known in the art, sensitive to degradation by exonucleases and therefore precludes loss of sensitivity of the assay method.

Further, said method reduces heme concentrations in the RNA-preparation below values inhibiting Taq-polymerase. This is because hemoglobin is removed, high heme concentrations decreasing the sensitivity of the assay method.

The term "immediately" means that the sample is lysed within seconds after

The term "immediately" means that the sample is lysed within seconds after removal from the patient in an agent depicted under (a). Said agent is preferably a guanidine salt such as guanidine is overlaid on a cushion of an RNAse-inhibiting agent depicted under (b). Said agent is preferably a cesium salt such as caesium trifluoroacetate. The Said agent is preferably a cesium salt such as caesium trifluoroacetate. The mRNA is advantageously obtained by ultracentrifugation through said cushion.

Preferably, the cancerous condition is related to prostate cancer, non-small or small cell lung cancer or sarcoma or malignant melanoma or breast cancer or colorectal cancer. The above enumeration of tumor types is to be understood as a selection of tumors that is recited as preferred examples. Other tumors may be also detected in their early stages in accordance with the present invention.

The figures show:

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Location of the specific oligonucleotide primers in the MAGE gene sequences. Grey boxes show the different exons. Arrows indicate the position of the oligonucleotides used for RT-PCR analyses. The expected size of the PCR products in the external as well as internal amplification is indicated.

Figure 2:

Number of BM aspirates from lung carcinoma patients with positive results in PCR-assay for at least one MAGE and CK-Immunocytochemistry.

#### Figur 3:

Number of prostate carcinoma patients with positive results in PCR-assay for at least one MAGE and PSA, and in CK-Immunocytochemistry in BM of at least one aspiration site.

#### Figure 4.

Number of prostate carcinoma patients with positive results in PCR-assay for at least one MAGE and PSA, and in CK-Immunocytochemistry in BM of at least one aspiration site.

- a) analysis related to primary tumor
- b) analysis related to progressive disease

#### Figure 5:

Mage-1 PCR assay of bone marrow samples from lung cancer patients. Patient samples 1-8 (ZI.JO., BA.MA., DE.IV., NE.WI., HO.FR., MA.JO., RE.LU., HE.JO) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 12. DNA ladder in lane M.

#### Figure 6:

Mage-2 PCR assay of bone marrow samples from lung cancer patients. Patient samples 1-8 (FI.RI., RE.LU., KU.LU., KL.JU., HE.JO., HA.AN., RO.AD., BE.FR.) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 12. DNA ladder in lane M.

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Mage-3 PCR assay of bone marrow samples from lung cancer patients. Patient samples 1-8 (HA.HI., DE.IV., HO.FR., MA.RO., LA.AL., AR.JO., VO.KL., WE.AL.) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 12. DNA ladder in lane M.

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Mage-4 PCR assay of bone marrow samples from lung cancer patients. Patient samples 1-6 (HA.AN., VO.KL., DE.IV., BE.FR., LA.BR., IN.FR..) in lane 3-8. Controls: mock preparation in lane 1, 10 LB23-SAR tumor cells in 1 ml blood in lane 9, 100 LB23-SAR tumor cells in 1 ml blood in lane 9, 100 LB23-SAR tumor cells in 1 ml blood in lane 9.

#### Figure 9:

Mage-12 PCR assay of bone marrow samples from lung cancer patients. Patient samples 1-8 (SO.JO., HA.HI., HO.FR., MA.JO., LA.BR., IN.FR., NI.OS. FI.RI.) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 12. DNA ladder in lane M.

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#### Figure 10:

Mage-1 PCR assay of bone marrow samples from prostate cancer patients and healthy persons. Patient samples 1-8 (FE.KU.re., RI.ER.le., ZO.JO.re., SC.HO.le., KM.Di.re., MA.GU.le., ME.JO.le., BE.WA.le.) in lane 4-11. Healthy persons in lane 12-15. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 16. DNA ladder in lane M.

#### Figure 11:

Mage-2 PCR assay of bone marrow samples from prostate cancer patients and healthy persons. Patient samples 1-8 (SC.GU.le., WO.JA.re., PF.LO.le., SC.AU.le., FE.KU.le., ZO.JO.le., ST.EB.le., SC.HO.le.) in lane 4-11. Healthy persons in lane 12-15. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane M.

#### Figure 12:

Mage-3 PCR assay of bone marrow samples from prostate cancer patients. Patient samples 1-8 (RI.ER.le., SC.GU.le, BA.GI.re., BE.WA.le, WO.JA.le., FE.KU.re., KN.DI.re., RI.FE.re.) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 12. DNA ladder in lane M.

#### Figure 13:

Mage-4 PCR assay of bone marrow samples from prostate cancer patients and healthy persons. Patient samples 1-8 (PF.LO.le., HI.ER.le., SC.HO.re., HE.RU.re., LU.VO.le., MA.GU.le., WI.JA.le., LE.DI.re.) in lane 4-11. Healthy persons in lane 12-15. Controls: mock preparation in lane 1, 10 LB23-SAR tumor cells in 1 ml blood in lane 2, 100 LB23-SAR tumor cells in 1 ml blood in lane 3, 1000 LB23-SAR tumor cells in 1 ml blood in lane M.

#### Figure 14:

MAGE-12 PCR assay of bone marrow samples from prostate cancer patients. Patient samples 1-8 (WO.JA.le., RI.ER.le., LU.VO.re., GE.CH.le., BA.Gl.re., WE.HJ.le., HI.ER.le., BI.GU.re.) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 12. DNA ladder in lane M.

#### Figure 15:

MAGE PCR assays were performed in quadruplicates. (a) Expression of MAGE-1. Tumor samples in lane 3-6. Controls: mock preparation in lane 8, tumor cell line Mz-2Mel in lane 1, 6, 7. DNA ladder in lane M. (b) Expression of MAGE-2 and -3. Corresponding to Mage-2, tumor samples in lane 4-7, tumor cell line Mz-2Mel in lane 2, 3 and 8, mock preparation in lane 12-15, tumor cell line Mz-2Mel in lane 10,11, and 16, mock preparation in lane 9. DNA ladder in lane Mz-2Mel in lane 10,11, and 16, mock preparation in lane 9. DNA ladder in lane Mz-2Mel in lane 4-7, tumor cell line LB23-5AR in lane 2,3 and 8, mock preparation in lane 1. Corresponding to MAGE-4, tumor samples in lane 12-15, tumor cell line Mz-2Mel in lane 4-7, tumor cell line Mz-2Mel in lane 16,11, and 16, mock lane 2,3 and 8, mock preparation in lane 1. Corresponding to MAGE-12, tumor samples in lane 12-15, tumor cell line Mz-2Mel in lane 10,11, and 16, mock preparation in lane 9. DNA ladder in lane Mz-2Mel in lane 10,11, and 16, mock preparation in lane 9. DNA ladder in lane Mz-2Mel in lane 10,11, and 16, mock preparation in lane 9. DNA ladder in lane Mz-2Mel in lane 10,11, and 16, mock

The examples illustrate the invention.

#### Example 1: Cell lines and Patients

In total, 28 tumor cell lines were tested for MAGE expression. Out of them 17 were of epithelial (MCF-7, BT20, SKBr3, MDA-MB: breast; LNCaP: prostate, SkCo, HT29, LS180, SW480: colon; A498, Caki I: kidney; HepB3, HepG2: liver;

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Panc-Tu: pancreas; Kato: stomach, A427: lung and A431: skin) and 2 were of mesenchymal origin (HT1080, LB23- SAR). 3 hematopoietic (U937, Raji,K562) and 6 tumor cell lines derived of neuroectodermal tissue (A172, U138: glioblastoma; Mel-Juso, Mel-Mei, A375, Mz-2Mel: melanoma) were analyzed. The melanoma cell line Mz-2Mel and the sarcoma cell line LB23-SAR were gifts of Francis Brasseur (Ludwig Institute, Brussels). The study population consisted of double-sided BM aspirates of 30 patients with prostate cancer (n=60 aspirates); single-sided BM aspirates were taken from 34 patients with non-small and small lung cancer (n=34 aspirates) and 6 patients with sarcoma (n=6 aspirates). Further, we analyzed peripheral blood samples from 12 patients with prostate cancer (n=12 samples), 6 patients with sarcoma (n=6 samples) and 12 patients with malignant melanoma (n=12 samples). All patients with prostate and lung cancer were staged as free of distant overt metastases (M0). In contrast, patients with sarcoma and malignant melanoma were suffering from advanced disease or metastases in different organs. Patients were graded by histopathological examination and staged according to the TNM-classification (4th edition, 1987). To test the specificity of the MAGE PCR assays, a "negative" control group consisting of 67 non-malignant patients: 20 BM aspirates and 20 peripheral blood samples with a history of trauma, benign tumors, inflammatory diseases or healthy donors were admitted to the study; furthermore, bone marrow of 27 healthy allogeneic bone marrow donors were analyzed with regard to expression of Mage gene products. Using heparine as an anticoagulant, BM was aspirated from one or two sides of the upper iliac crest under local anesthesia in patients with prostate cancer and sarcoma while chemotherapy on under general anesthesia in patients with lung cancer prior to tumorectomy.

#### **Example 2: Tissue sampling and preparation**

To avoid degradation of the RNA, 1 ml of the native sample was immediately within seconds mixed with 5 ml nucleic acid extraction buffer (4 M guanidine isothiocyanate, 0.5% sarcosyl (N-laurylsarcosine sodium salt), 25 mM sodium

and electrophoresis of PCR products. PCR carryover all samples were processed at a site separate from amplification preparation was also performed as described above. To minimize the potential of microscope and mixed in 1 ml peripheral blood of a healthy donor. RNA culture flasks, single cells of these lines were picked with a pipette under the sensitivity of the PCR assay, Mz-2Mel and LB23-SAR were harvested from tissue 4 minutes and dissolved in 5 µl DEPC-treated HPLC-water. 10 determine the night. After centrifugation the RNA pellet was washed with 70% ethanol, dried for isopropanol, 40 µl 3M sodium acetate, pH=5.0 and 20 µg glycogen at -20°C over with 300 µl chloroform. Subsequentially, the RNA was precipitated with 300 µl vortexing and centrifugation (1 min, 14,000 rpm) the upper phase was reextracted of a phenol-chloroform-isoamylalcohol mixture (25:24:1, vol:vol:vol). Again after HPLC-water. Sequentially, the sample was transferred to a fresh tube with 300 pl a bed of ice and the RNA pellet was dissolved in a total of 300 µl DEPC-treated the tube was cut off with a scalpel. The bottom was turned over again, placed on inverted, placed on a paper towel to drain for 2 minutes, and the bottom 1 cm of solution were removed by aspiration and discarded. The tubes were quickly nucleic acid extraction buffer/BM or blood mixture and the lower layer of CSTFAcentrifuged at 35,000 rpm for 18 h at 15°C. After centrifugation, the upper layer of diethyl pyrocarbonate (DEPC)-treated, autoclaved SW40 centrifuge tubes and trifluoracetate (Pharmacia, Freiburg, Germany) and 0.25 M EDTA, pH=7.0) in The lysate was gently overlaid onto a 5 ml cushion of CsTFA-solution (cesium For purifying total RNA the method of Okayama et al. was modified as follows: could easily and without risk of RNA degradation be stored at -20°C until needed. citrate, pH=7.0) and 0.7% 2-mercaptoethanol. After vigorous vortexing the sample

#### Example 3: Reverse-transcriptase polymerase chain reaction

Half of total RNA was denatured for 5 min at 70°C, quickly chilled on ice and was reverse transcribed using random h xamer primers (Boehringer Mannheim, Mannheim, Germany). The synthesis was carried out with a First-strand cDNA

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synthesis kit (Gibco, Eggenstein, Germany), including Superscript II (Gibco) in a final volume of 10 µl containing 50 mM Tris (pH=8.3), 75 mM Kcl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM total dNTP, 1.6 µg of the random primers and 100 units Superscript II. After the addition of RNA, the samples were incubated at 40°C for 1 hour and subsequently diluted with 10 µl HPLC-water.

PCR-reaction mixture (10 µl) was composed of 1 µl cDNA, 1 µl of 10xPCR buffer (100 mM Tris, pH=8.3, 500 mM KCl, 10 mM MgCl<sub>2</sub>), 40 µM dNTP, 0.4 µM of each of the two primers, 5 µg BSA (Boehringer Mannheim, Mannheim, Germany), 0.6 units of Taq DNA-Polymerase (Boehringer Mannheim, Mannheim, Germany) and overlaid with 12.5 µl of mineral oil. The primers for the MAGE genes were designed from previously published sequences (Tab. 1, Fig.1), and were selected to maximize mismatches between the different MAGE-sequences particularly in the 3'-region in order to avoid cross-amplification. Specific PCR assays were possible for MAGE genes 1,2,4 and 12 since the percentage of identity of these genes varies between 64 to 85 %. In contrast, the sequence of Mage-6 was found to be 99% identical to that of Mage-3. Therefore our primers for Mage-3/6 hybridize to both genes and PCR can detect Mage-3 and/or Mage-6 expression. Short primers in the range of 19-22 bases and annealing temperatures of about 60°C were most efficient. Also junction primers spanning over two exons showed surprising results in efficiency. As published recently, we applied primers for PSA (Tab. 1) on exons 2 and 3 that span a 1.6-kilobase intron and amplify three different PSA cDNAs. To confirm the presence of cDNA template in each sample, primers specific for p53 (kindly provided by Thomas Blankenstein, München, Tab. 1) were used to coamplify this ubiquitously expressed "housekeeping" gene. Oligonucleotide primers were synthesized and purified at Genset, Paris. For the external fragment amplification of the MAGE PCR assays following cycling profile was used: denaturation at 94°C for 6 min, annealing at 60°C for 30 sec and extension at 72°C for 2 min for the first cycle; denaturation at 93°C for 40 sec, annealing at 60°C for 30 sec and extension at 72°C for 20 sec for 14 cycles; denaturation at 93°C for 40 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec for 50 cycles; terminal extension at 72°C for 2 min and cooling at

of the nested PCR assay was 100%. ot a malignant tumor cell line mixed with 1 ml normal whole blood. Reproducibility approach, the RT-PCR assays for the different MAGE genes could detect 10 cells amplification products obtained was monitored with DNA sequencing. Using this Germany) was used as a reference marker for all assays. The specificity of the bromide staining under UV-light. A 1 kb DNA ladder (Gibco BRL, Eggenstein, on 1.8% agarose by gel electrophoresis and by direct visualization after ethidium preparation and RT-PCR were performed routinely. PCR products were analyzed contaminations. Negative control reactions (mock reactions) for the RNA amplification were performed in different rooms to avoid potential DNA crosslaminar air-flow bench with filtered pipette tips, external and nested fragment using plate control. Importantly, all pipetting was performed on ice under a was performed on a Hybaid Thermal Reactor (Biometra, Göttingen, Germany) 64°C and 7 cycles with 57°C, continuing 50 cycles with 57°C. The amplification and separating the 14 cycles into 7 cycles with and annealing-temperature of min. The cycling conditions for PSA varied by using conventional one-stage PCR 12°C (30 sec) with a final extension at 72°C for 2 min and cooling at 30°c for 1 amplification thirty more cycles were run at 93°C (40 sec), 58°C (30 sec) and tube containing the PCR mixture described above. For the nested fragment 30°C for 1 min. One microliter of the reaction was then transferred into a second

#### Example 4: Immunocytochemistry

After removal of 1 ml of whole BM and blood for PCR analysés, mononuclear cells (MNC) from the remaining aspirate were isolated by density gradient centrifugation through Ficoll-Hypaque (Pharmacia, Freiburg, Germany) at 400g for 30 minutes, interface cells were cytocentrifuged onto glass slides at 150g for 5 minutes (5x10<sup>5</sup> cells per slide). BM specimens were stained with monoclonal antibodies CK2 (kindly provided by Dr. M. Osborn, Max Planck Institut Göttingen, and later obtained from Dr. H. Bodenmüller, Boehring r Mannheim, Tutzing, and later obtained from Dr. H. Bodenmüller, Boehring r Mannheim, Tutzing, Germany; these as well as all other antibodies used in the experimental part of

the application were prepared according to conventional procedures. The specific antibodies used here are not essential for carrying out the invention) and A-45-B/B3 (kindly provided by Micromet GmbH, Martinsried, Germany), which detect common epitopes on cytokeratin components. The antibody reactions were developed with the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique combined with the Neufuchsin method for visualizing antibody binding. Briefly, after incubation with the primary antibody, a polyvalent rabbit anti-mouse tg antiserum (Z259, Dako, Hamburg, Germany), and performed complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase antibodies (D651, Dako, Hamburg, Germany) were used at the dilutions recommended by the manufacturer (Dakopotts, Hamburg, Germany). For each BM aspirate, two slides containing a total of 106 MNC were evaluated

### Example 5: Expression of MAGE genes in normal bone marrow and blood versus other markers

RNA was extracted from BM of control patients and subjected to RT-PCR amplification using primers from several histogenetic differentiation markers and tumor-associated antigens. (Tab. 2). After 50 cycles of amplification, product of all these candidate genes known to be expressed in malignancies was seen except PSA and MAGE genes. Nevertheless, PSA expression is restricted to malignancies of the prostate and seems to be downregulated in a fraction of disseminated tumor cells. In contrast, MAGE genes are ubiquitously expressed in malignancies and no amplification product after sensitive nested PCR was seen in any of the analyzed 20 BM aspirates and 20 peripheral blood samples. This result was confirmed when analyzing bone marrow aspirates of 27 healthy persons. None of said aspirates from the allogenic bone marrow donors expressed the MAGE genes, see Table 5.

The highly restricted expression pattern of tumor antigens in contrast to tumorassociated molecules strongly supports that MAGE genes may be interesting candidates for PCR-based detection of minimal residual disease.

### Example 6: Expression of MAGE genes in bone marrow samples of patients with different types of tumors

.4r Gelelectrophoresis of Mage PCR products is shown in examples in Figures 5 to ongin. Results and patient characteristics are listed in Table 4a-c and Table 7. blood samples from patients suffering from malignancies of different histological hematopoietic tissue encouraged us to evaluate BM aspirates and peripheral MAGE genes together with the complete absence of expression in normal showed MAGE-2 and -4 expression. This heterogenous expression pattern of the completely negative. Increasing the sensitivity by performing 65 cycles A498 therefore scored positive in the PCR. Only one renal cell carcinoma (A498) was with 35 cycles, 27 of 28 cell lines exhibited expression of at least one MAGE and hematopoietic and neuroectodermal origin (Tab. 3). Performing one-stage PCR RT-PCR assay in vitro and screened 28 cell lines of epithelial, mesenchymal, as shown for prostate cancer in Table 6, we initially evaluated our multimarker show a heterogenous expression pattern for different MAGE genes (1,2,3,4,6,12), disseminated tumor cells of different histological origin. Since primary tumors The objective of the study was to establish a PCR assay for detection of

#### Prostate cancer

First, BM sepirates were obtained from both sites of the iliac crest of patients with prostate cancer (Tab. 4a, Tab. 7). Out of the 30 patients transcripts of MAGE-1 were observed in 12 patients (40%), MAGE-2 in 3 pat. (17%), MAGE-3/6 in 5 pat. (17%), MAGE-4 in 6 pat. (20%) and MAGE-12 in 5 pat. (17%). The overall positivity for expression of at least one MAGE gene in one patient was 18 (60%). Interestingly, only two patients showed concordant expr ssion pattern of MAGE genes in both aspiration sites, while 16 patients displayed heterogenous

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expression. There was no correlation between the primary tumor stage, the histopathological grading or the mean PSA concentration in serum and the MAGE-positivity in the PCR-assay.

#### Lung cancer

The second epithelial tumor entity we were interested in was non-small and small lung cancer (Tab.4b). We analyzed single-sided BM aspirates of 34 carcinoma patients with positive results for MAGE-1 in 5 asp. (15%), MAGE-2 in 4 asp. (12%), MAGE-3/6 in 3 asp.(9%), MAGE-4 in 3 asp. (9%) and MAGE-12 in 4 asp.(12%). At least one MAGE expression was found in 12 asp. (35%). MAGE-PCR displayed positive signals in 7 of 19 BM aspirates of patients with squamous cell carcinoma and 3 of 10 analyzed BM aspirates of patients with adenocarcinoma.

#### Sarcoma

Since mesenchymal tumor cell lines express different MAGE genes, we analyzed single-sided BM aspirates of 6 sarcoma patients staged as M1 using the sensitive MAGE PCR assays (Tab. 4c). 5 samples showed at least expression of one MAGE gene, while MAGE 1,2,4,12 were positive in two tumor samples, respectively, and MAGE-3/6 was positive in one sample. Transcripts of all MAGE genes were found in BM aspirates of patient T.C., suffering from rhabdomyosarcoma with infiltrating bone metastases.

### Example 7: Correlation of MAGE expression with other tumor cell dissemination markers

All BM aspirates of patients with malignancies of the lung and prostate were evaluated by both the MAGE RT-PCR and the well established immunocytochemical assay using mAb against cytokeratins. Expression of at least one MAGE gene and CK-status of 34 analyzed lung carcinoma samples is displayed in Fig.2. 12 aspirates showed positive results in MAGE PCR assay, while 8 aspirat s revealed CK positive cells. 6 aspirates w re found to be concordant positiv and were all taken from patients with squamous cell

carcinoma. MAGE PCR showed positive results in 3 of 10 patients with adenocarcinoma, while immunocytochemistry exhibited no CK-positive cells in

this histological subtype. CK-immunocytochemistry and a third assay: a sensitive RT-PCR for PSA. Fig.3 shows the results of PCR assays for MAGE and PSA, and of CK-immunocytochemistry. As mentioned above, out of 30 patients with prostate carcinoma 18 patients showed expression for at least one MAGE gene. PSA expression was observed in 8 patients, while CK-immunostained cells were found in 9 patients. Interestingly, all PSA positive results of the MAGE PCR assay. Comparatively, CK-immunostained cells were found patients. Moreover, seven PSA- and CK-negative patients revealed MAGE PCR assay. Comparatively, CK-immunostained cells were found may be in four psa.

### samples: Expression in blood samples compared to bone marrow

Most of recently published PCR assays claim to detect circulating tumor cells and predict high sensitivity; nevertheless the results of the analyses of PB samples obtained with CK-immunocytochemistry are disappointing. We analyzed 18 blood samples of patients with prostate carcinoma (n=12) and sarcoma (n=6), that were taken in parallel to the BM aspiration. As listed in Table 4a, MAGE-1 and MAGE-3/6 transcripts were found in one patient, while MAGE-4 expréssion could be observed in two patients with prostate cancer. Interestingly, two patients with MAGE expression in blood exhibited a different pattern in bone marrow. PSA-lower sensitivity of the assay using peripheral blood was confirmed by analyzing patients with sarcoma (Tab. 4c). Only two patients revealed MAGE-2 and 3/6 expression, while the expression of the different MAGE genes in BM aspirates expression, while the expression of the different MAGE genes in BM aspirates could not be reproduced. Further, we analyzed 12 pati nts with advanced and could not be reproduced. Further, we analyzed 12 pati nts with advanced and

metastatic malignant melanoma using the sensitive MAGE RT-PCR assay. Expression of at least one MAGE gene was observed in only 5 patients with positive results for MAGE-1,2,3/6 and 12 in two samples and MAGE-4 in one sample. The failure to detect circulating cells in patients with localized or even advanced disease seems to indicate the bone marrow as preferential indicator compartment for detecting disseminated tumor cells.

### Example 9: Different expression of MAGE-genes in the primary tumor of prostate cancer patients

Total RNA was prepared primary tumors according to Chomczynski, Analytical Biochemistry 162 (1987) 156-159. Subsequently cDNA was synthesized as described in Example 6. As shown in Table 6, 7 of 20 tumors were positive for MAGE-1, 6 of 20 tumors for MAGE 2 and -12, respectively, 10 of 20 tumors for MAGE 3/6 and 4 of 20 for MAGE-4; overall, 14 of 20 tumors (70%) were positive for at least one MAGE gene product.

# Example 10: Different expression of MAGE-genes in the primary tumor and in the bone marrow of the same patient as determined by the method of the invention

Total RNA was prepared from the primary tumor of sarcoma patient H. M. (see also Tab. 4c) according to Chomczynski, Analytical Biochemistry 162 (1987) 156-159. Subsequently cDNA was synthesized as described in Example 3 followed by a one-step PCR with 35 cycles for MAGE 1, 2, 3/6, 4 and 12 as described in Example 6.

MAGE-analysis of a bone marrow sample of patient H. M. was carried out according to the method of the invention. Analysis of the primary tumor (results are shown in Figure 15) revealed predominant expression of MAGE-1 and only weak and inconsistent expression of other MAGE-gen s including MAGE-4 and 12. Analysis of the bone marrow, however, only revealed the expression of Mage-

4 and -12, but no expression at all of MAGE-1 (Tab. 4c). Thus, the MAGE-samily disseminated tumor cells does not necessarily match with that of the corresponding primary tumor. Therefore, therapeutic approaches according to claim 5, to eliminate systemically disseminated tumor cells thus preventing future distant metastasis should be preferably based on the analysis of the MAGE-expression pattern of said cells in an appropriate indicator tissue like bone marrow, rather than on analysis of the MAGE-expression pattern

#### Example 11: Risk analysis of prostate cancer patients

of the primary tumor.

analyzed, had progressive disease at the time of analysis as defined by an prostat clomy or radiation (results are shown in Fig. 4a). The remaining 8 patients neoadjuvant therapy of their primary tumor prior to curatively intended of the 30 patients, at the time of first diagnosis of prostate cancer or during data were exlucively taken from Table 7). MAGE-expression was analysed in 22 positive for both aspiration sites. For the risk analysis of prostate cancer patients SC.FR. and LE.DI., respectively; in addition, patient JU.HA. tumed out to be CKleft and negative results for the right bone marrow aspiration samples of patients reevaluation of the CK-immunocytochemistry data revealed positive results for the the time of first diagnosis of prostate cancer as given in Tab. 7. Finally, bone marrow aspiration, and therefore not identical with the initial PSA values at grading. Furthermore, the PSA values given in Tab. 4a are those at the time of data, there are some discrepancies to Table 7 with regard to tumor staging and listed as in Table 4a. However, as Table 4a contains more preliminary clinical listed in Table 7 and 4a (In Table 7 the same 30 prostate cancer patients are from the right and left iliac creat, respectively. Clinical data of the patients are -3/6, -4 or -12 was detectable in at least one of two bone marrow aspirates taken regarded MAGE-positive when at least one specific PCR-product for MAGE-1, -2, by bone marrow analysis according to the method of the invention. Patients were In 18 of 30 prostate cancer patients disseminated tumor cells could be detected

increasing blood concentration of PSA or by a local relapse; none of these patients, however, had clinical signs of distant metastasis at the time of MAGEanalysis. As it is well established in the art, these patients have a very high risk of future distant metastasis (Zietman, Cancer Supplement 71 (1993), 959-969; Fuks, Int. J. Radiation Oncology Biol. Phys. 21 (1991), 537-547; Pound, Urol. Clin. North Am 24 (1997), 395-406); thus it would be expected that most of them have already undergone systemic dissemination of cancer cells at the time of MAGEanalysis, when clinical evident distant metastasis has not yet developed but is likely to grow out of these disseminated single tumor cells in the future. Indeed, 7 of these 8 patients (87,5%) highly at risk of distant metastasis proved to be MAGE-positive. In contrast, only 11 of 22 patients (50,0%) analyzed in temporal association with their primary tumor, and thus representing both high and low risk patients according to future distant metastasis, were found to be MAGE-positive. Interestingly, only 22,7% of the patients in the latter group (5/22) were found to be MAGE-positive for both aspiration sites, compared to 50% in the high risk group (4/8). These data indicate, that detectable MAGE-expression in at least one bone marrow sample is associated with a high risk of future distant metastasis most probably due to the presence of systemically disseminated tumor cells. Thus, a subgroup of patients suffering from cancer and especially from prostate cancer being highly at risk of future distant metastasis may be identified by the method of the invention. This subgroup may be preferentially subjected to adjuvant cancer therapy according to claim 5 or any other adjuvant approach in order to prevent the outgrowth of distant metastases from systemically disseminated single cancer cells.

Table 1: Synthetic oligonucleotide primers for the nested RT-PCR amplification of the different MAGE genes and the conventional RT-PCR amplification of PSA and p53.

(g-51515-3)	ຊວາວາວຊຸຂວາ-່ີ ດັ	_
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ිccgtgagagagaaaggmc-3	$\delta$ '-ເວເ <u>ຮ</u> ູເຮູຂູຮູຊສູຊູຊູເລ-3'	MAGE-12
2,-८उ व व व द द द द द द द द द द द द द द द द	२,-१००१० ह्वाइव्यक्वहें	
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2,-८इर्बर्वटम्बेटइर्बर्टेटस्टर्ट-3	्र,-देइट्टव्यव्यड्डिट्टव्यड्डिट्टेट्र,	
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Table 2: Specificity of RT-PCR. Analyses of hematopoietic tissue from non-carcinoma control patients. Data are presented as positive bone marrow aspirates or PBL over total number of specimens assessed.

MARKER GENE	BONE	BLOOD
	MARROW	
· T		
i. Tumorassociated and histogenetic		
<u>differentiation markers:</u>		
CEA (Carcinoembryonic Antigen)	5/19	n.d.
erbB2	5/7	n.d.
erbB3	6/7	n.d.
EGP-40 (Epithelial Glycoprotein-40)	53/53	n.d.
Desmoplakin I	5/5	n.d.
		n.d. n.d.
Cytokeratin-18	5/7	
PIP (Prolactin-inducible Protein)	3/31	n.d.
PSM (Prostate-Specific Membrane	4/9	n.d.
Antigen)		
PSA (Prostate-Specific Antigen)	0/53	0/20
2. Tumorspecific Markers:	0/20	0/20
		•
MAGE - 1	0/20	0/20
MAGE - 2	0/20	0/20
	0/20	0/20
MAGE - 3/6	••	•
MAGE - 4	0/20	0/20
MAGE - 12	0/20	0/20

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Table 3: Expression of the different MAGE genes by malignant amor cell lines evaluated by specific RT-PCR amplification. Different levels of expression as deduced by band intensity of PCR products are represented by (+) or +. Absence of PCR product is indicated by -.

+	(+)	+	+	+	K 562 (erythroleukemia)
+		-	-	•	RAJI (Burkitt-lymphoma)
+	•	+	+	+	- (smohqmyl) YEQ U
(+)	+	+	•	+	LB-23 SAR (fibrosarcoma)
+	+	+	+	+	HT 1080 (fibrosarcoma)
					nigiro lamrəbozəm
					2. Tumors derived of
+	+	+	(+)	+	MZ-2 MEL (melanoma)
+	(+)	-	+	+	A 375 (melanoma)
+	- 3	+	+	+	MEL-MEI (melanoma)
+	+	+	+	+	MEL-JUSO (melanoma)
+	•	-	+	+	(smotssldoilg) 851 U
+	-	+	-	+	(glioblastoma)
					neuroektodermal origin
1				 	ld. Tumors derived of
	· · · · · · · · · · · · · · · · · · ·				(epidermoidcarcinoma)
+	+	+	+	+	I 54 A
+	+	+	+ .	÷	(gaul) 724 A
+	-	+	+	+	KATO (stomach)
+	(+)	-	+	+	PANC-TU (pancreas)
-	-	-	-	+	HEP G 2 (hepar)
+	•	+	+	+	HEP B 3 (hepar)
-	-	-	•	+	CaKi I (kidney)
-	-,	-	•	-	A 498 (Kidney)
+	-	•	•	-	SW 480 (colorectal)
+	+	+	+	+	LS 180 (colorectal)
+	(+)	+	+	+	HT 29 (colorectal)
(+)	-	+	•	+	SkCo (colorectal)
+	•	+	•	÷	LNCaP (prostate)
+	•	+	+	•	MDA-MB (breast)
+	-	+	•	+	2KBr 3 (preast)
÷	+	+	+	+	BT 20 (breast)
+	•	+		+	MCF-7 (breast)
					nigiro
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					IA. Tumors derived of
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-	-	-	-	-	
्राष्ट्रह	<b>Agalvi</b>	Mage	भ्रिष्ठिद्ध	Mage	

Tab.4: Clinical data and results (MAGE and PSA RT-PCR, CK-immunocytochemistry) on patients with a) prostate cancer

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ALYSES	MAGE-12		1						1		9											+			1		
BONE MARROW ANALYSES	MAGE-4		•			+	+		•		+									+	•	1	-		-		1
NONE M	MACE-376	1	•			+	+		+		•				+	,		+	1	•	+		•		1		
	MAGE-2		+						•	•	•			+	1	•	·	•	-	1		,					-
	MAGE-1		+	•	+	+	+	+	•	B	•	•	+	•	-		•	•	+			1	+		***************************************		•
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PSA		8.1		13		5.4		144		8.4		15.1		5.92		6.42		4.4		7.8		23.3	***************************************	2	*************	5.25	
TUMOR		3		2		2		3		÷		2		3		2		_		2		7		2		-	
TUMOR TUMOR SIZE CRADE		C / 113.F4		C / 1314		) I.I. / V	- 1	k1. / CI		C / 1314		11.1.7		C / 113/14		1.L./ V		1.1. / V		C / 1314		C / 13T4		N.C.1.7		V.I.2.I.7	
PATIENT		SC.AU.		SE.NI.	$\neg$	114.1118.		RI.ER.	コ	SC.FR.	コ	1,8.01.		.vo.jv.		11 E. WA.	_	CE.CH.	-	11 B. R.U.		KN.D1.		MB.JO.		JU.11A.	

I CARRTANSO OWN - MITHORIDIA

_	111.13R. D	-	BI.GU. B/	_	VK'IIV' C'.		WI.JA. C/		13 / C/1	H	MA.GU.   C/1314		70.JO.   C/1314		1.U.VO. C/1314		FB.KU. C/1374	-	SC.110. C/1314		ST.EU. A/TI		หเ.ศธ. 📗 🗛		KU.WI. A/		SC.GU.   C1.1314	_	SC.EU. C/		ા v/.Ll		YE.IIJ. D/	Tab. 4a (cor
	ו 21./ נו	_	13 / 172 2		C / 113114 2		C / 1314 2.		C / 13174 2		1314 2		13.1.4 2		13.14 2		1314 3		1314 3		7.1.1 2		7/TI 2		1 LL./ V		1314 2		C / 113.1/4	-	/T1 2		ם / 17   2	(continue)
	9.1		35.6		18.1		8.3	Ÿ	4.16		20.4		13	L	9.11		24		38.2		9.14		6.82		3.8		11.3		25	L	9.51		8.18	
	ח		Y		Y		Y		Y		y		Y						Y	٠.	)		y		у		Y		y		y		y	
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		-					n.d.		, , , , d.		n.d.		,	Ī	n.d.	_	<u> </u>	i	1		9.4.		1.4.	_			3.4.		n.d.		n.d.		n.d.	

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Tab.4: Clinical data and results (MAGE and PSA RT-PCR, CK-immunocytochemistry) on patients with b) lung cancer

	יוחו לם	o, iung cancer					-		
	PATIENT	HISTOLOGY	STAGING			HONE MARK	HONE MARROW ANALYSES		
				MACE-1	MAGE-2	MAGE-3/6	P-315VW	MAGE-12	CK-IMMUNC
_	VO.KL.	SOUAMOUS	TZNOMO	+	•	+	+	+	÷
	AR-JO.	DI.ASTOMA	OMINZL		-		•		÷
	WE.AL.	SOUAMOUS	OMINZI		•		•		÷
	RO.AD.	SOUAMOUS	TANOMO		+		•		-
	CA.AB.	SUOMAUS	UZNZI.	,	•		_	+	+
	F1.R1.	วาวร	OWINZI.	+	•			+	
	RB.LU.	ADENO	OWZNZI.			+	÷		
	118.30.	SUOMAUOS	OWINZL	•	+	1	•		÷
	KU.LU.	ONEICIV	T4N2M0			-	+	-	
	KL.JU.	ADENO	TINOMO	•		•	•		
	LÖ.MA.	ADENO	1'AN3M0	,	+		1		•
	IIA.AN.	ADENO	TINOMO			•	-		
	CR.GO.	SOUAMOUS	OWONZI.	•	+			-	+
	DE.IV.	SOUAMOUS	TINIM0	+	t	÷	•		÷
	BR.FR.	SOUAMOUS	OMINZI					•	
	MA.RO.	SOUAMOUS	TZNOMO	•				-	
	114.111.	ADENO	OMONZI.	•		,			•
	110.FR.	SUOMAUOS	TAN3MO	•		•	•		
	MA.10.	ADIENO	OMINZI.		•				
	LA.BR.	SOUAMOUS	TZNIMO	1		8			
	IN.FR.	SARCOMA	OMONEL		•	-	-		•
	1,Λ.Λί	ADIENO	OMZNZI.		_		•		
	SC.10.	ADENO	12N2M0	•	•	•	•		
	21.30.	SQUAMOUS	OMINGL	+ ,			1		•
	KR.WI.	SQUAMOUS	IZNIMO	•	•	•	•		
	NI.OS	LARGIS-CILL	DANOMO	+	•	1	•	+	-
	SO.JO.	SQUAMOUS	TZNZMO	•	•	•			
	NE.WI.	SOUAMOUS	UZNZNI.	-	•				-
	BA.MA.	SOUAMOUS	TANIMO	•	*	•	•	-	
	138.58.	SOUAMOUS	TZNOMO	t	•				

Tab. 4b (continue)

sc.jo.	PO.MA.	IV.MA.	MA.RI.
ADENO	LARGE-CELL	SOUVMORS	SOUVMOUS
TZNIMO	TZNIMO	TZNOMO	T3N2M0
	•	•	•
	•	•	¢
		•	•
•	•	•	•
	•	•	•
•			٠

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Tab.4: Clinical data and results of MAGE RT-PCR on patients with c) sarcoma

		÷	<b>}</b> -	-	$\overline{}$		<del>,</del>	_
PH ANALYSES				*	1		•	NAAC10: 0
	MAGIE-12		-	•	-	-	+	+
ALYSES	MAGE-4			-			+	+
HONE MARROW ANALYSES	MAGE-3/6			•	•			+
BONE	MAGE-2	-	•		•	+	1	+
	MAGE-1		•		+		•	+
M-STAGE		Ξ	Ē	MO	Æ	æ	MO	Σ
LOCALIZATION OF M-STAGE PRIMARY TUMOR		RIGHT FEMUR	RIGHT FEMUR	PARAVERIEBRAL	PELVIS MINOR	MULTILOCULAR	CARDIAC STOMACII	PELVIS MINOR
IIISTOLOGY		SYNOVIAL SARCOMA	LEIOMYOSARCOMA	BPITHELOID SARCOMA	LEIOMYOSARCOMA	EWING'S SARCOMA	UNDIFFERENTIATED SARCOMA	VWODIVSOXWOGIIVHII
PATTENT		I.G.	R.T.	R.R.	L.G.	D.K.	11.M.	T.L.

PCR of total number of specimens assessed. bone marrow donors). Data are presented as negative and positive results in RT-Tabl 5: Analyses of bone marrow aspirates of 27 healthy persons (= allog neic

WAGE-12	0	72
W∀@E¬ŧ	0	72
W¥GE-3\8	0	72
WYGE-2	0	72
MAGE-1	0	72
	PCR-pos.	PCR-neg.
WYKKEK GENE	BONE MAR	WOS

**Tabl** 6: Expression of the different MAGE genes by primary tumors of 20 patients with prostate cancer. Evaluation was obtained by specific RT-PCR amplification of native malignant prostatic tissue. Data are presented as negative and positive results in RT-PCR of total number of specimens assessed.

MARKER GENE	PROSTATI	E TUMOR
	PCR-pos.	PCR-neg.
MAGE-1	7	13
MAGE-2	6	14
MAGE-3/6	10	10
MAGE-4	4	16
MAGE-12	6	14
at least one MAGE	14	6

RNSDOCID: <WO 9848788A2 1 >

NR 1297	· >	×	+	+	. +	· †	· <b>†</b>	+	C.16	+	Adeno / 2	T2NoMo/p	5/1996	₩	BL.GU.
NR 11/9/	>	×	: +	: +	. +	. +	. +	+	14.0	+	Adeno / 2	T,NoMo/p	8/1995	C	AK.HA.
BCK+LK 96	· >	0	+	+	+	+	<b>+</b>	<u>'</u>	5.31	+	Adeno / 2	T,N,M,/p	7/1995	ი	WLJA.
NR 5/97	. W	×	+	<u>+</u>	+	+	+	+	4.2	+	Adeno / 1	TJNoMo/p	3/1996	ဂ	BA.GI.
NR 1/98	>	×	<b>!</b>	<i>+</i>	<i>-</i>	-/-	<b>-</b>	+	20.4	+	Adeno / 0	T <sub>0</sub> N <sub>0</sub> M <sub>0</sub> /p	5/1996	ဂ	MA.GU.
iBCR 3/98	>	×	<b>‡</b>	<b>+</b>	<u>+</u>	<u>+</u>	+		13.0	÷	Adeno / 2	T,NoMo/p	1/1996	ဂ	zo.jo.
NR 10/97	>	×	‡	<b>-</b>	<u>+</u>	<b>+</b>	<u>+</u>		14.5	+	Adeno / 2	T,N <sub>0</sub> M <sub>0</sub> /p	7/1996	Ġ	LU.VO.
n.a.	>	×	+	-	+	<del>'</del>	<del> </del>		23.8	+	Adeno / 3	TJNoMo/p	7/1996	ი	FE.KU.
BCR 3/98	>	×	<b>‡</b>	<del>+</del>	<u>+</u>	<u>+</u>	+/-		56.3	<u>+</u>	Adeno / 3	T,NoMo/p	8/1995	C	SC.HO.
BCR 3/98	C	×	<b>†</b>	<u>+</u>	<u>+</u>	<b>-</b>	<del>-</del> /-	<u>+</u>	9.14	<u>+</u>	Adeno / 2	T,N,M,/1	5/1996	>	ST.EB.
iBCR 1/98	>	×	<b>+</b>	<u>+</u>	<u>+</u>	+	<b>†</b>	+	41.4	<b>‡</b>	Adeno / 2	T,N,M,/p	3/1996	<b>A</b>	RI.FE.
LR+DM 97	দ	×	<u>+</u>	-/-	<u>+</u>	<u>-</u>	<u>+</u>	<u>+</u>	0.777	+	Neuro / 3	T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /p	4/1996	ი	KU.WI.
LR 11/97	×	× 	+	+	<u>+</u>	<u>+</u>	<u>-</u> /-	+	15.7	+		T,N,M,/p	10/1995	>	sc.cu.
BCR + LR 96	≯	0	<u>+</u>	-/-	+	<u>+</u>	<u>-</u>	<u>+</u>	n.a.	+	Adeno / 2	T,NoMo/p	1992	ი —	SC.EU.
n.a.	A	×	<u>+</u>	<u>+</u>	<u>+</u>	<u>-</u>	<u>-</u> -	<u>+</u>	9.27	+	Adeno / 2	T,NoMo/p	12/1995	ဂ	PF.LO.
n.a.	n.a.	×	+	+	<del>-</del>	<u>+</u>	<u>+</u>	<u>+</u>	8.2	<b>\</b>	Adeno / 2	T <sub>2</sub> N <sub>6</sub> M J b	7/1996	₩.	WE.HJ.
NR 1/98	>	×	<u>+</u>	<u></u>	<u>'</u>	<u>+</u>	<u>+</u>	<u>+</u>	5.25	<b>‡</b>	Adeno / 2	T2NoMo/p	7/1996	ဂ	JU.HA.
NR 1/98	₩	×	+	+	<u>-</u> -	<u>+</u>	+		134	+	Adeno / 2	T,N,M,/b	7/1996	ဂ	MEJO.
BCR + LR /96	>	0	+	<u>-</u>	<u>+</u>	<u>+</u>	+-		п.а.	+	Adeno / 2	T,N,M,/p	10/1992	ဂ	KN.DI.
BCR 6/96	Q	0	<i>-</i>	<del>'</del>	<b>†</b>	<u>+</u>	<u>+</u>	+	n.a.	+	Adeno / 3	T,N <sub>0</sub> M <sub>0</sub> /p	7/1994	ဂ	HE.RU.
LR 96, DM 97	Ħ	0	<b>-</b> -	<u></u>	<u>+</u>	<u>+</u>	<u></u>	<u>+</u>	n.a.	<u>+</u>	Adeno / 3	T,N,M <sub>0</sub> /p	1992	ဂ	GE.CH.
NR 3/97	A	×	-/-	<u></u>	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	9.51	<u>+</u>	Adeno / 2	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub> /p	10/1995	>	BE.WA.
BCR 5/96	ם	0	<del>-</del>	<u>+</u>	<u>+</u>	<del>'</del>	<u>+</u>	<u>+</u>	n.a.	<u>+</u>	Adeno / 3	T,NoMo/b	12/1991	₩	WCJA.
n.a.	A	×	-/-	<u>+</u>	<u>+</u>	<u>+</u>	<b>+</b>		15.1	<b>‡</b>	Adeno / 0	T,N,M,/p	n.a.	₩	LE.DI.
BCR 11/95	A	0	<b>-</b>	<b>*</b>	<u>+</u>	<u>+</u>	<u>+</u>	<del>'</del>	9.9	<b>‡</b>	Adeno / 1	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub> /p	6/1994	ဂ	SC.FR.
BCR 96 DM 97	D	×	<b>-</b> /-	<u></u>	+	<u>+</u>	<del>'</del>		144.4	<b>‡</b>	Adeno / 3	T,N2M0/b	12/1994	ם	RLER.
NR 1/98	Þ	×	<u>+</u>	<b>‡</b>	++	<u>-</u>	<b>+</b>		5.4	<b>;</b>	Adeno / 2	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub> /p	9/1995	₩	BA.HE.
NR 11/97	>	×	<u> </u>	<u>+</u>	<u>+</u>	<u>+</u>	+,		11.6	<del> </del>	Adeno / 2	T,N,Mo/p	6/1996	ဂ	SE.NI.
BCR 5/96	Α	0	-/-	-/-	-/-	-/+	+/-		11.8	<b>*</b>	Adeno / 2	T,N <sub>0</sub> M <sub>0</sub> /p	12/1994	ဂ	SC.AU.
			Ur	Ur	Ur	Ur	Ur	Ur.	(	\$					
			12	4	3/6	2	7		(ngn)				,		
				į					Level			07246	diagnosis	0,000	
dn-wond	ment	aspiration	Sinen sa	inalyses neg	bone marrow r CK-Analys MAGE Genes	RIGITOR M		2 3	PS 4	2	Histology/	Stage	fire of	Cunical	Pattent
Fallan	7	30			3		-1			22					

#### Table 7-legend:

Clinical data of 30 patients with prostate cancer and the corresponding results of MAGE RT-PCR, PSA RT-PCR and CK-Immunocytochemistry as obtained by the analysis of bone marrow samples from the right and left iliac crest, respectively. Table lists: patients, clinical stage A-D, time of first diagnosis, histology (adenocarcinoma = adeno, neuroendocrine carcinoma = neuro) and corresponding grading (1=well differentiated, 2= moderately differentiated, 3= poorly differentiated), TNM-stage related to biopsy and lymphadenectomy (= b) or related to prostatectomy and lymphadenectomy (= p), PSA-level prior to therapy (= initial PSA), results of immunocytochemistry and RT-PCR, time of aspiration (0 = bone marrow aspiration at the time of progressive disease but without clinically evident distant metastasis, x = bone marrow aspiration at the time of first diagnosis of prostate cancer or during neoadjuvant therapy of the primary tumor prior to curatively intended prostatectomy or radiation), treatment modality (A = neoadjuvant antiandrogen therapy and subsequent prostatectomy, B = neoadjuvant antiandrogen therapy and subsequent radiation, C = transurethral resection, D = primary antiandrogen therapy, E = prostatectomy and adjuvant antiandrogen therapy, F = neoadjuvant antiandrogen therapy plus chemotherapy, and subsequent prostatectomy, G = prostatectomy), as well as time and result of last clinical follow-up ( NR = no relapse, BCR = biochemical relapse as defined by continuously PSA levels over prolonged periods of time, iBCR = initially increasing PSA level over a short period of time prior to end of follow-up, LR = local relapse, DM = clinically evident distant metastasis). n.a. = not available.

SEGUENCE LISTING

(I) GENEEAT INFORMATION:

(E) BORLYT CODE (SIB): 85125

(E) CONNIKA: GELWSUN (D) SIMIE: none

- (C) CILX: Planegg
- (B) SIREET: Am Klopferspitz 19

of disseminated tumor cells

(C) OPERATING SYSTEM: PC-DOS/MS-DOS (B) COMPUTER: IBM PC compatible (A) MEDIUM TYPE: Floppy disk

(A) DESCRIPTION: \desc = "oligonucleotide"

\desc = "oligonucleotide"

(D) SOFTWARE: Patentin Release #1.0, Version #1.30 (EPO)

(ii) TITLE OF INVENTION: Novel primers and methods for the detection

(ii) MOLECULE TYPE: other nucleic acid

(x) SEGUENCE DESCRIPTION: SEQ ID NO: 1:

(ii) MOLECULE TYPE: other nucleic acid

(D) TOPOLOGY: linear (C) STRANDEDNESS: single (B) TYPE: nucleic acid (A) LENGTH: 21 base pairs (1) SEQUENCE CHARACTERISTICS:

(S) INFORMATION FOR SEQ ID NO: 2:

(iii) HYPOTHETICAL: YES

(Y) DESCRIBLION:

(S) INFORMATION FOR SEQ ID NO: 1:

(iv) COMPUTER READABLE FORM:

(iii) NUMBER OF SEQUENCES: 20

(D) TOPOLOGY: linear (C) **SIKYNDEDNESS**: sindle (B) TYPE: nucleic acid (A) LENGTH: 21 base pairs (i) SEQUENCE CHARACTERISTICS:

O AADDAADOOD DOTTDADATD

ZZ

- - - (A) NAME: Micromet GmbH
      - (i) APPLICANT:

- (iii) HYPOTHETICAL: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CAGGAGCTGG GCAATGAAGA C

21

- (2) INFORMATION FOR SEQ ID NO: 3:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 22 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear

  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CATTGAAGGA GAAGATCTGC CT

22

- (2) INFORMATION FOR SEQ ID NO: 4:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 22 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GAGTAGAAGA GGAAGAAGCG GT

22

- (2) INFORMATION FOR SEQ ID NO: 5:
  - (i) SEQUENCE CHARACTERISTICS:

- (xt) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
  - (iii) HYPOTHETICAL: YES
- (Y) DESCEIDATION: \quad \
  - (D) TOPOLOGY: linear
  - (C) STRANDEDNESS: single
    - (B) LXbE: uncleic sciq
  - (A) LENGTH: 21 base pairs
  - (i) SEQUENCE CHARACTERISTICS:
  - (S) INFORMATION FOR SEQ ID NO: 7:

20

- I CASSTANSO NW. - TINOTOM

AADDDDADTD DTDTDADTAD

- (x;) SEĞNENCE DESCKIBLION: SEĞ ID NO: 0:
  - (iii) HYPOTHETICAL: YES
- (ii) MOFECUTE LABE: oCHEL uncjeic sciq
  - (D) TOPOLOGY: Linear
  - (C) SIRANDEDNESS: single
    - (B) TYPE: nucleic acid
  - (A) LENGTH: 20 base pairs
  - (I) SEQUENCE CHARACTERISTICS:
  - (S) INEOKWATION FOR SEQ ID NO: 6:

81

**DOTOBBAD ODBBDDDAAD** 

- (x;) SEĞNENCE DESCKIBLION: SEĞ ID NO: 2:
  - (iii) HYPOTHETICAL: YES
- (ii) WOFECUTE TYPE: other nucleic scid
  (ii) MOFECUTE TYPE: other nucleic scid
  - (D) LODOFOGK: Jinear
  - (C) SIKYNDEDNESS: sjudje
    - (B) TYPE: nucleic acid
  - (A) LENGTH: 18 base pairs

CACCAAGGAG AAGATCTGCC T

21

- (2) INFORMATION FOR SEQ ID NO: 8:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 21 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TCCTCAGTAG TAGGAGCCTG T

21

- (2) INFORMATION FOR SEQ ID NO: 9:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 20 base pairs
      - (B) TYPE: nucleic acid
      - (C) STRANDEDNESS: single
      - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCCGTGAGGA GGCAAGGTTC

20

- (2) INFORMATION FOR SEQ ID NO: 10:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 22 base pairs(B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"

- (i) SEQUENCE CHARACTERISTICS:
- (S) INFORMATION FOR SEQ ID NO: 13:

20

CTGGGCAATG AAGACCCACA

- (xŢ) SEĞNENCE DESCKIBLION: SEĞ ID NO: 15:
  - (iii) HYPOTHETICAL: YES
- (A) DESCRIPTION: \desc = "oligonucleotide" (ii) MOLECULE TYPE: other nucleic acid
  - (D) LOBOFOCK: Jinear
  - (C) **SIKYNDEDNESS**: sindje
  - (B) TYPE: nucleic acid
  - (A) LENGTH: 20 base pairs
  - (i) SEQUENCE CHARACTERISTICS:
  - (S) INFORMATION FOR SEQ ID NO: 12:

20

TAGAGTTCGG CCGAAGGAAC

- (x;) SEGNENCE DESCRIBTION: SEG ID NO: 11:
  - (fff) HABOLHELICAT: KEZ
- (A) DESCRIPTION: \desc = "oligonucleotide"
  - (ii) MOLECULE TYPE: other nucleic acid
    - (D) TOPOLOGY: linear
    - (C) STRANDEDNESS: single
      - (B) TYPE: nucleic actd
    - (A) LENGTH: 20 base pairs
    - (I) SEGUENCE CHARACTERISTICS:
    - (S) INFORMATION FOR SEQ ID NO: 11:

AT SASABASSTTO ASTITUS TA

(xt) SEGUENCE DESCRIPTION: SEQ ID NO: 10:

(iii) HYPOTHETICAL: YES

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CATTGAAGGA GAAGATCTGC CT

22

- (2) INFORMATION FOR SEQ ID NO: 14:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 20 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
    - (ii) MOLECULE TYPE: other nucleic acid
      - (A) DESCRIPTION: /desc = "oligonucleotide"
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CAGGCTTGCA GTGCTGACTC

. 20

- (2) INFORMATION FOR SEQ ID NO: 15:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 19 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - . (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

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(D) TOPOLOGY: linear (C) SIEVADEDNESS: sindle (B) TYPE: nucleic acid (A) LENGTH: 21 base pairs (i) SEQUENCE CHARACTERISTICS:

(xt) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

(ii) MOLECULE TYPE: other nucleic acid

(xŢ) SEĞNENCE DESCKIBLION: SEĞ ID NO: IE:

(D) LODOFOCK: Jinear (C) STRANDEDNESS: single (B) TYPE: nucleic acid (A) LENGTH: 21 base pairs (i) SEQUENCE CHARACTERISTICS:

(S) INFORMATION FOR SEQ ID NO: 17:

(iii) HABOLHELICYF: XE2

AADDDDADTD DTDTDADTAD

(A) DESCRIPTION: \desc = "oligonucleotide"

(A) DESCRIPTION: \desc = "oligonucleotide"

(S) INEOKWYLION EOK SEÖ ID NO: 18:

CACCAAGGAG AAGATCTGCC T

(iii) HYPOTHETICAL: YES

- (i) SEQUENCE CHARACTERISTICS:
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(ii) MOLECULE TYPE: other nucleic acid

- (A) LENGTH: 20 base pairs

(D) TOPOLOGY: linear

- (S) INFORMATION FOR SEQ ID NO: 16:
  - 6T
  - **GEACOSTAN** GEAGGCAAG

88L97/86 OM **LCL/Eb88/05081** 

- (iii) HYPOTHETICAL: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CAGGCTTGCA GTGCTGACTC T

21

- (2) INFORMATION FOR SEQ ID NO: 19:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 20 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TCCGTGAGGA GGCAAGGTTC

20

- (2) INFORMATION FOR SEQ ID NO: 20:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 19 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide" /
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GAGCCTGCGC ACCCACCAA

19

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#### CLAIMS

primers having the same 3' portion as one of the following groups (i) strand thereof, said primer being selected from the group of a MAGE tumor-specific antigen or a part thereof or to a complementary complementary to the messenger RNA transcribed from a gene encoding molecule A primer specifically hybridizing to a nucleic acid

of primers:

5'-tccgtgaggaggcaaggttc-3' 5'-atcggattgactccagagagta-3'	(SEƠ ID NO: 10)
5'-caccaaggagaagatctgcct-3' 5'-tcctcagtagtaggagcctgt-3'	(SEQ ID NO: 8)
5'-gaageceggeceaggeteg-3' - 6'-gatgaetetggteaggeaa-3'	(2EG ID NO: 9)
e,-dadfagaagaggaggcggf-3' 6'-cattgaaggagagactgcct-3'	(SEG ID NO: 4)
5'-දෑ අදුරුද්ද අදුරුණු අදුරුණු දිරුණු දිරුණු දිරුණු දිරුණු අදුරුණු අදුරුණු අදුරුණු අදුරුණු අදුරුණු අදුරුණු අද වේදා අදුරුණු	(SEQ ID NO:2)

(SEQ ID NO: 20)

(SEQ ID NO: 19)

(SEQ ID NO: 18)

(SEQ ID NO: 17)

(SEQ ID NO: 16)

(SEQ ID NO: 15)

(SEQ ID NO: 14)

(SEQ ID NO: 13)

(SEO ID NO: 12)

(SEQ ID NO: 11)

5'-gagcctgcgcaccaa-3'

5'-tccgtgaggaggcaaggtc-3'

5'-caggcttgcagtgctgactct-3'

5'-gatgactctggtcagggcaa-3'

2,-88ccc88ccs88-3,

5-caggcttgcagtgctgactc-3

5'-cattgaaggagagatctgcct-3'

5'-ctgggcaatgaagacccaca-3'

5'-tagagttcggccgaaggaac-3'

2,-csccssddsdssdstctdcct-3,

G

(a)

(q)

;and

- (ii) primers that overlap with a primer sequence depicted in (a) or (b).
- 2. The primer according to claim 1 which is a primer depicted in either group (a) or group (b).
- 3. A diagnostic composition comprising at least 4 primers according to claim 1 or 2 wherein said at least four primers hybridize pair-wise to strands of opposite orientation of at least two different nucleic acid molecules wherein strands of one orientation are complementary to the mRNA transcribed from the genes of at least two different MAGE tumor-specific antigens.
- 4. A method of detecting disseminated tumor cells indicative of a cancerous condition in a patient comprising:
  - (a) carrying out PCR on cDNA obtained from mRNA from one or more patient samples using at least two primers according to claim 1 or 2 hybridizing pair-wise to strands of opposite orientation of at least one cDNA molecule wherein strands of one orientation are complementary to the mRNA transcibed from the genes of at least one MAGE tumor-specific antigen; and
  - (b) detecting one or more PCR products.
- 5. A method of preparing a tumor adjuvant vaccine comprising the following steps:
  - (a) carrying out PCR on cDNA obtained from mRNA from one or more patient samples using at least four primers according to claim 1 or 2 hybridizing pair-wise to strands of opposite orientation of at least two different nucleic acid molecules wherein strands of one orientation are complementary to the messenger RNA transcribed

from the genes of at least two different MAGE tumor-specific antigens;

- (b) detecting resulting PCR products; and
- (c) using at least one MAGE-gene product, expression of said MAGE-gene product, expression of said MAGE-gene product, expression of said MAGE-antigen-derived peptides resembling MHC-restricted T-cell-epitopes, whole MAGE-antigens or fragments thereof, MAGE-transfected host cells, DNA vaccinating strategies using MAGE-encoding nucleotide sequences or any other immunization method based on MAGE-gene products or their coding nucleotide sequences.
- 6. The method according to claim 4 or 5 wherein said cDNA is obtained by:
  (a) preparing mRNA from one or more samples removed from said
- (b) reverse transcribing said mRNA.

patient; and

- 7. The method according to any one of claims 4 to 6 wherein said PCR is nested PCR.
- S. The method according to any one of claims 4 to 7, wherein said primers hybridize to 2 to 6 different nucleic acid molecules complementary to the mRNA transcribed from the genes of MAGE tumor-specific antigens and the complementary strands thereof.
- 9. The method according to any one of claims 4 to 8 wherein said MAGE tumor-specific antigens are the MAGE -1, -2, -3, -4, -6, or -12 tumor-specific antigens.
- The method according to any one of claims 4 to 9 wherein said samples
   are removed from two different aspiration sites.

- 11. The method according to any one of claims 4 to 10 wherein said samples are bone marrow aspirates.
- 12. The method according to any one of claims 4 to 11, wherein said mRNA preparation comprises the following steps:
  - (a) immediately lysing the sample in buffer essentially completely avoiding RNA-degradation; and
  - (b) optionally centrifuging the mRNA obtained in the lysate through a cushion of an RNAse-inhibiting agent, preferably cesium trifluoroacetate.
- 13. The method according to any one of claims 4 to 12 wherein said cancerous condition is related to prostate cancer, non-small or small lung cancer, sarcoma, malignant melanoma, breast cancer or colorectal cancer.

external as well as internal amplification is indicated Fig. 1: Location of the specific oligonucleotide primers in the MAGE gene sequences. Grew boxes show the different exons. Arrows indicate the position of the oligonucleotides used for RT-PCR analyses. The expected size of the PCR products in the

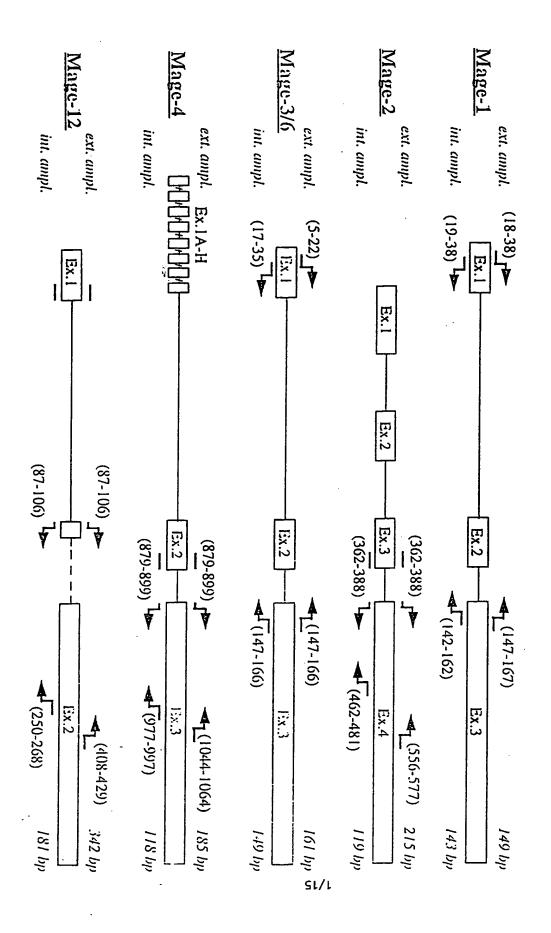
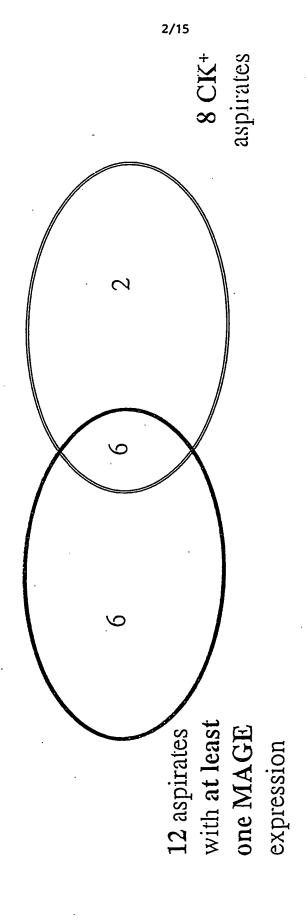


Fig.2: Number of BM aspirates from lung carcinoma patients with positive results in PCR-assay for at least one MAGE and in CK-Immunocytochenistry:



 $\Sigma = 34$  aspirates analysed

Fig. 3:. Number of prostate carcinoma patients with positive results in PCR-assay for at least one MAGE and PSA, and in CK-Immunocytochemistry in BM of at least one aspiration site.

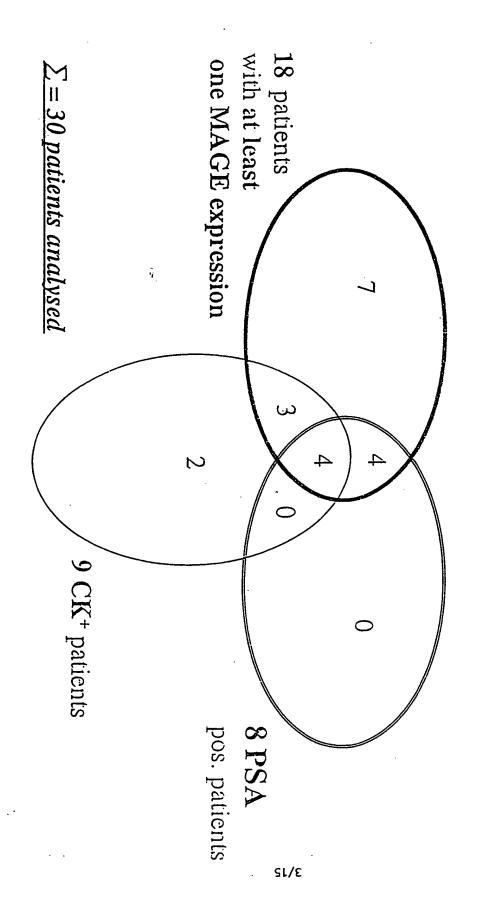
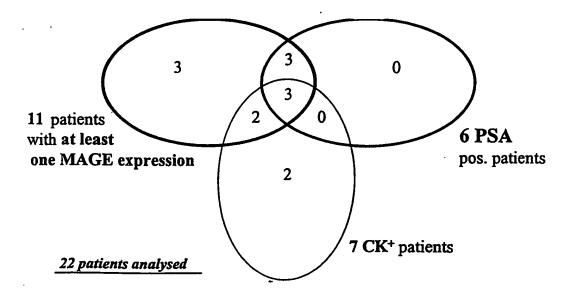


Fig. 4:. Number of prostate carcinoma patients with positive results in PCR-assay for at least one MAGE and PSA, and in CK-Immunocytochemistry in BM of at least one aspiration site

### a) analysis related to primary tumor



### b) analysis related to progressive disease

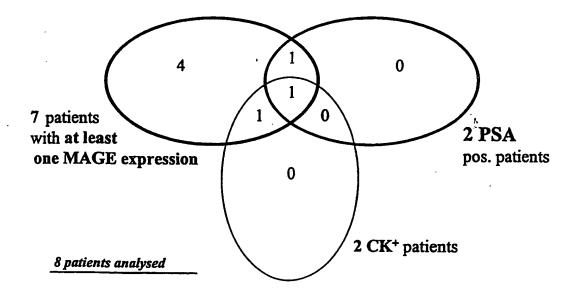
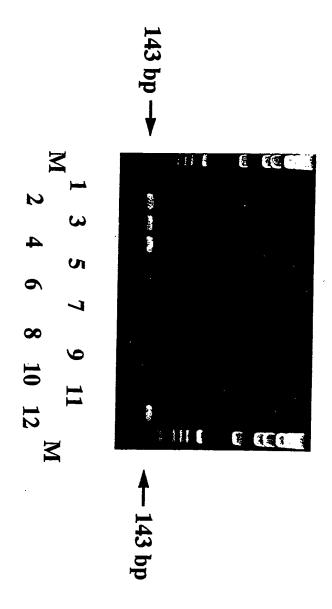


Figure 5

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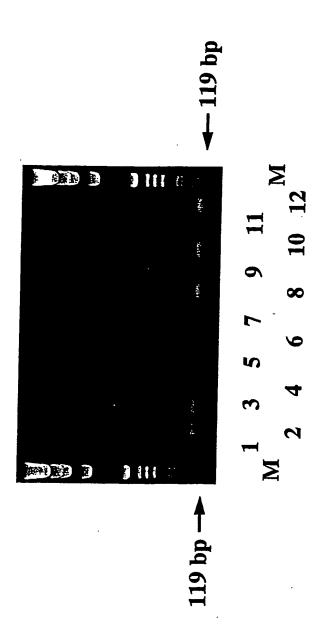
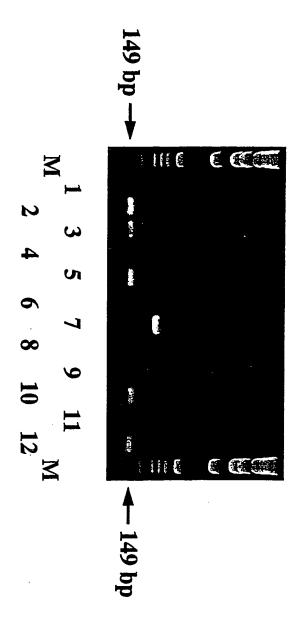


Figure 6

### Figure 7



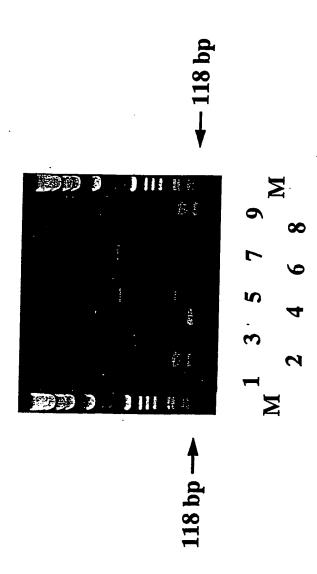
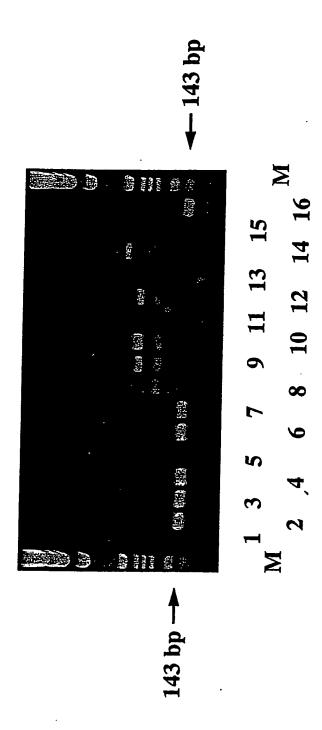


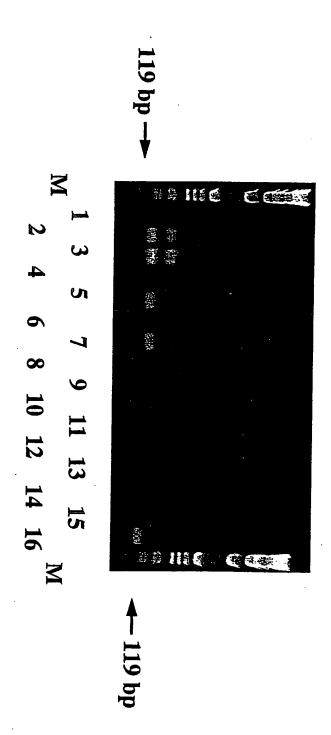
Figure 8

Figure 9

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· Figure 10



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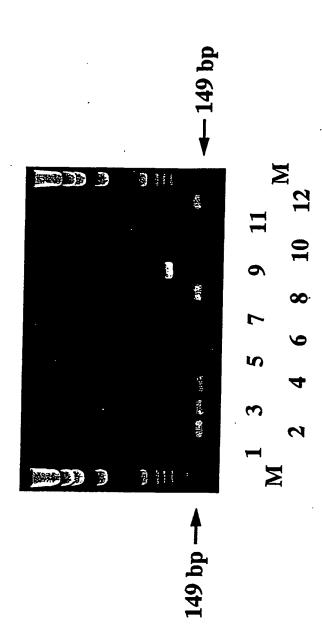


Figure 12

Figure 13

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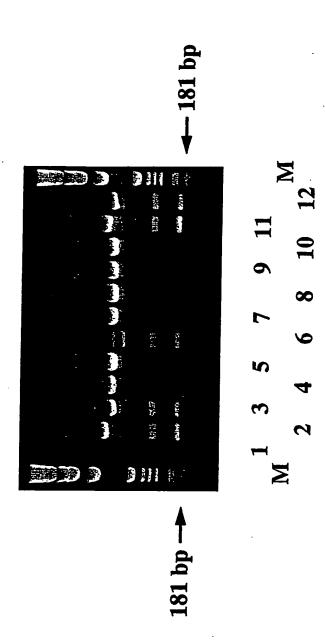


Figure 14

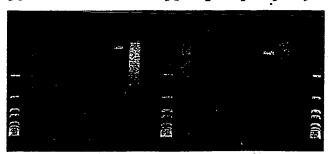
### Figure 15

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### INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> :	A3	(11) International Publication Number: WO 98/46788
C12Q 1/68, G01N 33/50	AS	(43) International Publication Date: 22 October 1998 (22.10.98
(21) International Application Number: PCT/EP (22) International Filing Date: 9 April 1998 ( (30) Priority Data: 97106026.4 11 April 1997 (11.04.97) (34) Countries for which the regional or international application was filed: (71) Applicant (for all designated States except US): MIC GMBH [DE/DE]; Am Klopferspitz 19, D-82152 (DE). (72) Inventors; and (75) Inventors/Applicants (for US only): KUFER, Peter [Graf-Konrad-Strasse 2, D-85368 Moosburg ZIPPELIUS, Alfred [DE/DE]; Klobensteinerstring D-81545 München (DE). (74) Agent: VOSSIUS & PARTNER; Postfach 86 07 67, I München (DE).	DE et a	BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published  With international search report.  (88) Date of publication of the international search report: 28 January 1999 (28.01.99)

(54) Title: PRIMERS AND METHODS FOR THE DETECTION OF DISSEMINATED TUMOR CELLS

#### (57) Abstract

The present invention relates to primers that specifically hybridize to nucleic acid molecules complementary to the messenger RNA transcribed from genes encoding MAGE tumor-specific antigens or a part thereof or to a complementary strand thereof encoding MAGE tumor-specific antigens as well as to diagnostic compositions comprising said antigens. The present invention further relates to methods for detecting disseminated tumor cells employing the primers of the invention as well as methods for preparing a tumor adjuvant vaccine.

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	tion searched other than minimum documentation to the extent th		
Electionic d	lata base consulted during the international search (name of data	base and, where practical, e	earch terms used)
C. DOCUM	ENTS CONSIDERED TO BE RELEVANT		
Category 3	Citation of document, with indication, where appropriate, of the	relevant passages	Relevant to claim No.
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	Z#21 YL0050\814\	
	vol. 88, no. 10, Part I, Suppl., page	
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	micrometastatic epithelial cancer cells in	
	reverse-transcriptase polymerase chain reaction analyses for detection of	
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	1-5,7,9; figures 9-13,17-20; examples	
	see page 1, line 1 - line 14; claims	
	see abstract	
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		^
	* see in particular page 2110, column 2, paragraph 2 *	
	column 2, paragraph 5; figure 1; table 1	
	page 2111, column 1, paragraph 2 - see page 2112, column 1, paragraph 2 -	
	see page 2109, column 1, paragraph 1 -	
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	vol. 13, no. 8, 1995, pages 2109-2116,	
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#### (54) Title: PRIMERS AND METHODS FOR THE DETECTION OF DISSEMINATED TUMOR CELLS

#### (57) Abstract

The present invention relates to primers that specifically hybridize to nucleic acid molecules complementary to the messenger RNA transcribed from genes encoding MAGE tumor-specific antigens or a part thereof or to a complementary strand thereof encoding MAGE tumor-specific antigens as well as to diagnostic compositions comprising said antigens. The present invention further relates to methods for detecting disseminated tumor cells employing the primers of the invention as well as methods for preparing a tumor adjuvant vaccine.

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#### PRIMERS AND METHODS FOR THE DETECTION OF DISSEMINATED TUMOR CELLS

The present invention relates to primers that specifically hybridize to nucleic acid molecules complementary to the messenger RNA transcribed from genes encoding MAGE tumor-specific antigens or a part thereof or to a complementary strand thereof as well as to diagnostic compositions comprising said antigens. The present invention further relates to methods for detecting disseminated tumor cells employing the primers of the invention as well as methods for preparing a tumor adjuvant vaccine.

Immunotherapeutic approaches have previously been applied to patients with minimal residual disease and could reduce mortality and recurrence associated with distant metastases. Since early occult dissemination of tumor cells is already present in about half of the cancer patients, efficient treatment strategies have to be developed for eliminating residual tumor cells. Many immunocytochemical and PCR-based assays using histogenetic differentiation markers exclusively allow the detection of micrometastatic cells that worsen the prognosis after local tumorectomy without identifying antigens for adjuvant immunotherapy. Limitations of RT-PCR analyses in oncological disorders resulted in the tissue-specific rather than tumor-specific expression of marker genes. Detection of disseminated tumor cells is thus restricted to a few, certain tumor types deriving from similar histological origin. Moreover, tumor cells are only detectable in front of special tissue background, so that most published approaches depend on samples taken from mesenchymal compartments. The presence of ubiquitous transcriptional factors and minimal activation of promotor sequences in background cells may cause illegitimate transcription and reduce specificity and sensitivity due to

competing background signals. Furthermore, most assays measure only a single marker gene not considering inter- and intra-individual tumor cell heterogenity.

Several fumor rejection antigens have recently been characterized that are presented on major histocompatibility complex (MHC) class I molecules to autologous cytolytic T-lymphocytes (CTL). The MAGE genes belong to a family of 12 closely related genes with an overall homology of 64-85% and are classified as "cancer-testis-antigens".

The specific primers as well as the regions they prime to in the MAGE genes are, The specific primers as well as the regions they prime to in the MAGE genes are, however, inappropriate for the diagnosis of disseminated tumor cells in human samples. WO 96/29430 discloses genetic markers for the detection of melanomas and breast cancer cells including occult cancer cells or metastatic cells derived therefrom. In said application, it is further described that a number of markers including MAGE-3 may be employed to detect such tumors. However, methods disclosed in this application are not expected to be suited for the detection of disclosed in this application are not expected to be suited for the detection of histological origins.

Since, in particular, the early reliable detection of disseminated fumor cells in a patient enables the early treatment and/or vaccination in order to prevent recurrence of cancerous growth, the technical problem underlying the present invention was to improve the sensitivity of the prior art methods for detecting such disseminated tumor cells in patients with maligancies of many different histological origins. The solution to said problem is achieved by the embodiments characterized in the claims.

Accordingly, the present invention relates to a primer specifically hybridizing to a nucleic acid molecule complementary to the messenger RNA transcribed from a

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gene encoding a MAGE tumor-specific antigen or a part thereof or to a complementary strand thereof, said primer being selected from the group of

(i) primers having the same 3' portion as one of the following groups of primers:

(a)

5'-gtagagttcggccgaaggaac-3'	(SEQ ID NO:1
5'-caggagctgggcaatgaagac-3'	(SEQ ID NO:2)
5'-cattgaaggagaagatctgcct-3'	(SEQ ID NO: 3)
5'-gagtagaagaggaagaagcggt-3'	(SEQ ID NO: 4)
5'-gaagccggcccaggctcg-3'	(SEQ ID NO: 5)
5'-gatgactctggtcagggcaa-3'	(SEQ ID NO: 6)
5'-caccaaggagaagatctgcct-3'	(SEQ ID NO: 7)
5'-tcctcagtagtaggagcctgt-3'	(SEQ ID NO: 8)
5'-tccgtgaggaggcaaggttc-3'	(SEQ ID NO: 9)
5'-atcggattgactccagagagta-3'	(SEQ ID NO: 10)
(b)	
5'-tagagttcggccgaaggaac-3'	(SEQ ID NO: 11)
5'-ctgggcaatgaagacccaca-3'	(SEQ ID NO: 12)
5'-cattgaaggagaagatctgcct-3'	(SEQ ID NO: 13)
5'-caggcttgcagtgctgactc-3'	(SEQ ID NO: 14)
5'-ggctcggtgaggaggcaag-3'	(SEQ ID NO: 15)
5'-gatgactctggtcagggcaa-3'	(SEQ ID NO: 16)
5'-caccaaggagaagatctgcct-3'	(SEQ ID NO: 17)
5'-caggcttgcagtgctgactct-3'	(SEQ ID NQ: 18)
5'-tccgtgaggaggcaaggttc-3'	(SEQ ID NO: 19)
5'-gagcctgcgcacccaccaa-3'	(SEQ ID NO: 20)

and;

(ii) primers that overlap with a primer sequence depicted in (a) or (b).

The term "overlap" in this context means the overlap of at I ast one nucleotide.

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Accordingly, said primers can hybridize to RNA, preferably mRNA or to DNA, preferably cDNA.

In accordance with the present invention, it was found that the primers having the above-recited 3' ends are particularly useful in detecting disseminated turnor cells of many different histological origins at a sensitivity level that was unknown from the prior art. Additionally, primers that overlap with the above-recited nucleic acid sequences and that hybridize to MAGE genes have been found useful for the detection of disseminated turnor cells in human samples. Preferably, said primers overlap with the nucleic acid sequences depicted under (a), above. It is further preferred that the primers of the invention have a length of between 15 and 25 nucleotides. With the exception of primers that recognize MAGE-3 and -6, each of asid primers specifically detects the expression of only one MAGE gene.

occurs under stringent conditions. Such conditions can be devised by the person skilled in the art according to conventional protocols. The specific hybridization antigens in a sensitive RT-PCR. Such RT-PCRs can be devised by the person not cross-amplify nucleic acids from the genes of other MAGE tumor specific accordance with the present invention is intended to mean that these primers do enough for such an analysis. The term "specifically hybridizing" as used in primers has, in accordance with the present invention, proven not to be high malignancies of many different histological origins since the sensitivity of said suitable for detecting disseminated tumor cells in samples of patients with the MAGE genes to which the primers described in the prior art hybridize are not be emphasized that the prior art methods and primers as well as the regions of primers, even if the analyzed patient has no clinical evidence of disease. It has to cells of many different histological origins can be detected using the above-recited by the present invention that, in contrast to prior art reports, disseminated tumor of patients where disseminated tumor cells are detected. It was surprisingly found condition at a very early stage of the disease and thus allows the timely treatment The present invention accordingly provides a means for detecting a cancerous

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skilled in the art without undue burden according to conventional protocols; see, e.g., "Nucleic Acid Hybridization, A Practical Approach"; edited by Hames & Higgins, IRL Press, Oxford 1985.

The primers of the invention are advantageously used in PCR amplification of nucleic acid sequences derived from the desired MAGE genes or fragments thereof. The primers of the invention are devised to avoid the amplification of genomic DNA but to amplify a DNA sequence derived from the reverse transcription of messenger RNA. In this way, the PCR product can be unambiguously correlated with expressed MAGE-genes. PCR may be carried out according to conventional methods. Also, the PCR product may be detected according to protocols that are established in the art.

Preferably, the primer of the invention is a primer depicted in either group (a) or group (b), supra.

The primers of this embodiment have been particularly useful and advantageous in identifying disseminated tumor cells in samples of patients with malignancies of many different histological origins. It is also particularly preferred that primers of group (a) are used as primers for a first round of PCR amplification whereas primers of group (b) are used in a nested primer PCR amplification.

The present invention further relates to a diagnostic composition comprising at least four primers according to the invention wherein said at least four primers hybridize pair-wise to strands of opposite orientation of at least two different nucleic acid molecules wherein strands of one orientation are complementary to the messenger RNA transcribed from the genes of at least two different MAGE tumor-specific antigens.

The diagnostic composition of the invention is particularly useful for carrying out a variety of PCRs and thus for unambiguously identifying the presence of disseminated tumor cells in patients with malignancies of many different histological origins. As has been found in accordance with the present invention,

at least two different MAGE-genes from the group of MAGE-1,-2,-3,-4, -6 and -12 should be checked for expression in order to further secure the diagnostic value of the investigation. Preferably, said diagnostic kit comprises at least one pair of primers specific for each of the MAGE-1,-2,-3,-4, -6 and -12 genes. It is further preferred that the analysis of the sample is carried out with the kit of the invention until the expression of at least two different MAGE-genes has been secured.

The bottling of the primers in said kit is effected according to conventional procedures. Preferably, each primer is separately bottled. Alternatively, the primers specific for each MAGE-gene are bottled together with the understanding that primers for a primary and a nested primer reaction are separately bottled.

The present invention further relates to a method of detecting disseminated tumor

cells indicative of a cancerous condition in a patient comprising:

(a) carrying out PCR on cDNA obtained from mRNA from one or more patient samples using at least two primers of the invention hybridizing pair-wise to atrands of opposite orientation of at least two one cDNA molecule wherein strands of one orientation are complementary to the messenger RNA stranscribed from the genes of at least one MAGE tumor-specific antigen;

(b) detecting one or more PCR products.

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The term "cancerous condition" is intended to mean the whole spectrum from the initiation of malignant transformation in single cells to advanced cancer disease including distant solid metastasis.

Preferably, at least four primers specific for at least two MAGE transcripts and complementary strands thereof are employed in the method of the invention.

Additionally, the present invention relates to a method of preparing a tumor adjuvant vaccine comprising the following at ps:

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(a) carrying out PCR on cDNA obtained from mRNA from one or more patient samples using at least four primers of the invention hybridizing pair-wise to strands of opposite orientation of at least two different nucleic acid molecules wherein strands of one orientation are complementary to the messenger RNA transcribed from the genes of at least two different MAGE tumor-specific antigens;

- (b) detecting resulting PCR products; and
- (c) using at least one MAGE-gene product, expression of said MAGE-gene being detected as a result of step (b) for preparing an adjuvent tumor vaccine based on MAGE-antigen-derived peptides resembling MHCrestricted T-cell-epitopes, whole MAGE-antigens or fragments thereof, MAGE-transfected host cells, DNA vaccinating strategies using MAGEencoding nucleotide sequences or any other immunization method based on MAGE-gene products or their coding nucleotide sequences.

This embodiment of the invention relates to the direct application of the positive analysis of the presence of disseminated tumor cells in a human sample. Once said tumor cells have been detected, a suitable antidote for the further proliferation of said tumor cells eventually resulting in tumorous growth should be devised. The method of the present invention provides such an antidote. The tumor adjuvant vaccine can be prepared according to conventional protocols. In particular, it is envisaged to pulse dendritic cells isolated from the patient with peptides derived from the coding sequences of those MAGE-genes detected to be expressed by the disseminated tumor cells of this particular patient using the diagnostic composition of the present invention, these peptides being suited for binding to MHC-molecules of this patient thus resembling MHC-restricted T-cells epitopes. The peptide-pulsed dendritic cells will then be reinjected into the patient and will migrate to the T-cell zones of secondary lymphoid organs where they prime MAGE-specific T-lymphocytes thus resulting in an immune response against MAGE-positive tumor cells.

In a preferred embodiment of the method of the invention, said cDNA is obtained

- by: breparing mRNA from one or more samples removed from said patient;
- (b) reverse transcribing said mRNA.The preparation of said mRNA and the reverse transcription thereof is

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ine preparation of said mRNA and the reverse transcription thereof is conveniently effected by conventional procedures.

Reverse transcription of mRNA resulting in the corresponding cDNA may be carried out by random hexanucleotide priming as described in Example 3 by oligo-dT-priming or by specific priming using one or more oligonucleotides complementary to one or more MAGE-mRNA species. Due to the high degree of sequence homology among different members of the Mage gene family, specific primers for the cDNA-synthesis may be identified that efficiently hybridize to the primers for the cDNA-synthesis may be identified that efficiently hybridize to the mRNA of more than one Mage gene and most preferably to all the Mage genes used according to the present invention (Mage-1, -2, -3/6, -4 and -12). Such "pan-Mage primers" can be identified and tested according to the state of the art manuals e.g. Sambrook (Molecular Cloning; A Laboratory Manual, 2<sup>nd</sup> Edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour Laboratory Press, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY (1989)) or Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY (1989)) or DC dold Spring Harbour Laboratory Press, Cold Spring Harbour, NY (1989)) or DC dold Spring Harbour Laboratory Press, Cold Spring Harbour, NY (1989)).

In an additional preferred embodiment of the method of the invention, said PCR is

nested PCR.

As has been found in accordance with the present invention, the nested PCR is a further safeguard for obtaining unambiguous results with regard to the patient's disease status.

In a further preferred embodiment of the method of the invention, said primers hybridize to 2 to 6 different nucleic acid molecules complementary to the

messenger RNA transcribed from the genes of MAGE tumor-specific antigens and to complementary strands thereof.

In a further preferred embodiment of the method of the invention, said MAGE tumor-specific antigens are the MAGE-1, -2, 3, -4, -6 or -12 tumor specific antigens.

Of all MAGE antigens, the above-recited antigens most often appear on tumor cells, as was found in accordance with the present invention, and are therefore particularly indicative of the risk of cancer to develop metastatic tumors.

It is further preferred in accordance with the present invention to remove samples from two different aspiration sites. As was found in accordance with the present invention, the analysis of double-sided aspiration significantly improves sensitivity. It is therefore advisable to use double-sides aspiration in the routine diagnosis of tumor cell dissemination.

In a further preferred embodiment of the method of the invention, said samples to be analyzed are bone marrow aspirates.

As has been surprisingly found in accordance with the present invention, the use of bone marrow aspirates as a source for analyzing the metastatic potential of a patient is more sensitive than using peripheral blood as suggested by the prior art.

Additionally preferred is a method wherein said mRNA preparation comprises the following steps:

- (a) immediately lysing the sample in buffer essentially completely avoiding RNA-degradation; and
- (b) optionally centrifuging the mRNA obtained in the lysate through a cushion of an RNAse-inhibiting agent, preferably cesium trifluoracetate.

This embodiment of the invention is advantageous since it precludes the loss of mRNA that is, as is well known in the art, sensitive to degradation by exonucleases and therefore precludes loss of sensitivity of the assay method.

Further, said method reduces heme concentrations in the RNA-preparation below values inhibiting Taq-polymerase. This is because hemoglobin is removed, high heme concentrations decreasing the sensitivity of the assay method.

The term "immediately" means that the sample is lysed within seconds after removal from the patient in an agent depicted under (a). Said agent is preferably a guanidine salt such as guanidine isothiocyanate. After lysis of the sample, said sample is overlaid on a cushion of an RNAse-inhibiting agent depicted under (b). Said agent is preferably a cesium salt such as caesium trifluoroacetate. The Said agent is preferably a cesium salt such as caesium trifluoroacetate. The MRNA is advantageously obtained by ultracentrifugation through said cushion.

Preferably, the cancerous condition is related to prostate cancer, non-small or small cell lung cancer or sarcoma or malignant melanoma or breast cancer or colorectal cancer. The above enumeration of tumor types is to be understood as a selection of tumors that is recited as preferred examples. Other tumors may be also detected in their early stages in accordance with the present invention.

The figures show:

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Location of the specific oligonucleotide primers in the MAGE gene sequences. Grey boxes show the different exons. Arrows indicate the position of the PCR oligonucleotides used for RT-PCR analyses. The expected size of the PCR products in the external as well as internal amplification is indicated.

#### Figure 2:

Number of BM aspirates from lung carcinoma patients with positive results in PCR-assay for at least one MAGE and CK-Immunocytochemistry.

#### Figure 3:

Number of prostate carcinoma patients with positive results in PCR-assay for at least one MAGE and PSA, and in CK-Immunocytochemistry in BM of at least one aspiration site.

#### Figure 4.

Number of prostate carcinoma patients with positive results in PCR-assay for at least one MAGE and PSA, and in CK-Immunocytochemistry in BM of at least one aspiration site.

- a) analysis related to primary tumor
- b) analysis related to progressive disease

#### Figure 5:

Mage-1 PCR assay of bone marrow samples from lung cancer patients. Patient samples 1-8 (ZI.JO., BA.MA., DE.IV., NE.WI., HO.FR., MA.JO., RE.LU., HE.JO) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 12. DNA ladder in lane M.

#### Figure 6:

Mage-2 PCR assay of bone marrow samples from lung cancer patients. Patient samples 1-8 (FI.RI., RE.LU., KU.LU., KL.JU., HE.JO., HA.AN., RO.AD., BE.FR.) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 12. DNA ladder in lane M.

#### Figure 7:

Mage-3 PCR assay of bone marrow samples from lung cancer patients. Patient samples 1-8 (HA.HI., DE.IV., HO.FR., MA.RO., LA.AL., AR.JO., VO.KL., WE.AL.) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 12. DNA ladder in lane M.

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Mage-4 PCR assay of bone marrow samples from lung cancer patients. Patient samples 1-6 (HA.AN., VO.KL., DE.IV., BE.FR., LA.BR., IN.FR..) in lane 3-8. Controls: mock preparation in lane 1, 10 LB23-SAR tumor cells in 1 ml blood in lane 9, 100 LB23-SAR tumor cells in 1 ml blood in lane 9, 100 LB23-SAR tumor cells in 1 ml blood in lane 9.

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Mage-12 PCR assay of bone marrow samples from lung cancer patients. Patient samples 1-8 (SO.JO., HA.HI., HO.FR., MA.JO., LA.BR., IN.FR., NI.OS. FI.RI.) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 12. DNA ladder in lane M.

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#### Figure 10:

Mage-1 PCR assay of bone marrow samples from prostate cancer patients and healthy persons. Patient samples 1-8 (FE.KU.re., RI.ER.le., ZO.JO.re., SC.HO.le., KM.Dl.re., MA.GU.le., ME.JO.le., BE.WA.le.) in lane 4-11. Healthy persons in lane 12-15. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2M I tumor cells in 1 ml blood in lane 16. DNA ladder in lane 3, 1000 Mz-2M I tumor cells in 1 ml blood in lane 16. DNA ladder in lane M.

#### Figure 11:

Mage-2 PCR assay of bone marrow samples from prostate cancer patients and healthy persons. Patient samples 1-8 (SC.GU.le., WO.JA.re., PF.LO.le., SC.AU.le., FE.KU.le., ZO.JO.le., ST.EB.le., SC.HO.le.) in lane 4-11. Healthy persons in lane 12-15. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane M.

#### Figure 12:

Mage-3 PCR assay of bone marrow samples from prostate cancer patients. Patient samples 1-8 (RI.ER.le., SC.GU.le, BA.GI.re., BE.WA.le, WO.JA.le., FE.KU.re., KN.DI.re., RI.FE.re.) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tumor cells in 1 ml blood in lane 12. DNA ladder in lane M.

#### Figure 13:

Mage-4 PCR assay of bone marrow samples from prostate cancer patients and healthy persons. Patient samples 1-8 (PF.LO.le., HI.ER.le., SC.HO.re., HE.RU.re., LU.VO.le., MA.GU.le., WI.JA.le., LE.DI.re.) in lane 4-11. Healthy persons in lane 12-15. Controls: mock preparation in lane 1, 10 LB23-SAR tumor cells in 1 ml blood in lane 2, 100 LB23-SAR tumor cells in 1 ml blood in lane 3, 1000 LB23-SAR tumor cells in 1 ml blood in lane M.

#### Figure 14:

MAGE-12 PCR assay of bone marrow samples from prostate cancer patients. Patient samples 1-8 (WO.JA.le., RI.ER.le., LU.VO.re., GE.CH.le., BA.Gl.re., WE.HJ.le., HI.ER.le., BI.GU.re.) in lane 4-11. Controls: mock preparation in lane 1, 10 Mz-2Mel tumor cells in 1 ml blood in lane 2, 100 Mz-2Mel tumor cells in 1 ml blood in lane 3, 1000 Mz-2Mel tum

#### Figure 15:

MAGE PCR assays were performed in quadruplicates. (a) Expression of MAGE-1. Tumor samples in lane 3-6. Controls: mock preparation in lane 8, tumor cell line Mz-2Mel in lane 1, 6, 7. DNA ladder in lane M. (b) Expression of MAGE -2 and -3. Corresponding to Mage-2, tumor samples in lane 4-7, tumor cell line Mz-2Mel in lane 2, 3 and 8, mock preparation in lane 1. Corresponding to MAGE-3, tumor samples in lane 10,11, and 16, mock preparation in lane 12-15, tumor cell line Mz-2Mel in lane 10,11, and 16, mock preparation in lane 9. DNA ladder in lane Mz-2Mel in lane 4-7, tumor cell line LB23-5AR in lane 2,3 and 8, mock preparation in lane 1. Corresponding to MAGE-4, tumor samples in lane 4-7, tumor cell line LB23-5AR in lane 2,3 and 8, mock preparation in lane 12-15, tumor lane 12-15, tumor lane 9. DNA ladder in lane 1. Corresponding to MAGE-12, tumor samples in lane 9. DNA ladder in lane 9. DNA ladder in lane Mz-2Mel in lane 10,11, and 16, mock preparation in lane 9. DNA ladder in lane Mz-2Mel in lane 10,11, and 16, mock preparation in lane 9. DNA ladder in lane Mz-2Mel in lane 9. DNA ladder in lane 9.

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The examples illustrate the invention.

#### Example 1: Cell lines and Patients

In total, 28 tumor cell lines were tested for MAGE expression. Out of them 17 were of epithelial (MCF-7, BT20, SkBr3, MDA-MB: breast; LNCaP: prostate, SkCo, HT29, LS180, SW480: colon; A498, Caki I: kidney; HepB3, HepG2: liver;

Panc-Tu: pancreas; Kato: stomach, A427: lung and A431: skin) and 2 were of mesenchymal origin (HT1080, LB23- SAR). 3 hematopoietic (U937, Raji,K562) and 6 tumor cell lines derived of neuroectodermal tissue (A172, U138: glioblastoma; Mel-Juso, Mel-Mei, A375, Mz-2Mel: melanoma) were analyzed. The melanoma cell line Mz-2Mel and the sarcoma cell line LB23-SAR were gifts of Francis Brasseur (Ludwig Institute, Brussels). The study population consisted of double-sided BM aspirates of 30 patients with prostate cancer (n=60 aspirates); single-sided BM aspirates were taken from 34 patients with non-small and small lung cancer (n=34 aspirates) and 6 patients with sarcoma (n=6 aspirates). Further, we analyzed peripheral blood samples from 12 patients with prostate cancer (n=12 samples), 6 patients with sarcoma (n=6 samples) and 12 patients with malignant melanoma (n=12 samples). All patients with prostate and lung cancer were staged as free of distant overt metastases (M0). In contrast, patients with sarcoma and malignant melanoma were suffering from advanced disease or metastases in different organs. Patients were graded by histopathological examination and staged according to the TNM-classification (4th edition, 1987). To test the specificity of the MAGE PCR assays, a "negative" control group consisting of 67 non-malignant patients: 20 BM aspirates and 20 peripheral blood samples with a history of trauma, benign tumors, inflammatory diseases or healthy donors were admitted to the study; furthermore, bone marrow of 27 healthy allogeneic bone marrow donors were analyzed with regard to expression of Mage gene products. Using heparine as an anticoagulant, BM was aspirated from one or two sides of the upper iliac crest under local anesthesia in patients with prostate cancer and sarcoma while chemotherapy of under general anesthesia in patients with lung cancer prior to tumorectomy.

#### **Example 2: Tissue sampling and preparation**

To avoid degradation of the RNA, 1 ml of the native sample was immediately within seconds mixed with 5 ml nucleic acid extraction buffer (4 M guanidine isothiocyanate, 0.5% sarcosyl (N-laurylsarcosine sodium salt), 25 mM sodium

and electrophoresis of PCR products. PCR carryover all samples were processed at a site separate from amplification preparation was also performed as described above. To minimize the potential of microscope and mixed in 1 ml peripheral blood of a healthy donor. RNA culture flasks, single cells of these lines were picked with a pipette under the sensitivity of the PCR assay, Mz-2Mel and LB23-SAR were harvested from tissue 4 minutes and dissolved in 5 µl DEPC-treated HPLC-water. To determine the night. After centrifugation the RNA pellet was washed with 70% ethanol, dried for isopropanol, 40 µl 3M sodium acetate, pH=5.0 and 20 µg glycogen at -20°C over with 300 µl chloroform. Subsequentially, the RNA was precipitated with 300 µl vortexing and centrifugation (1 min, 14,000 rpm) the upper phase was reextracted of a phenol-chloroform-isoamylalcohol mixture (25:24:1, vol:vol:vol). Again after HPLC-water. Sequentially, the sample was transferred to a fresh tube with 300 µl a bed of ice and the RNA pellet was dissolved in a total of 300 µl DEPC-treated the tube was cut off with a scalpel. The bottom was turned over again, placed on inverted, placed on a paper towel to drain for 2 minutes, and the bottom 1 cm of solution were removed by aspiration and discarded. The tubes were quickly nucleic acid extraction buffer/BM or blood mixture and the lower layer of CSTFAcentrifuged at 35,000 rpm for 18 h at 15°C. After centrifugation, the upper layer of diethyl pyrocarbonate (DEPC)-treated, autoclaved SW40 centrituge tubes and trifluoracetate (Pharmacia, Freiburg, Germany) and 0.25 M EDTA, pH=7.0) in The lysate was gently overlaid onto a 5 ml cushion of CsTFA-solution (cesium For purifying total RNA the method of Okayama et al. was modified as follows: could easily and without risk of RNA degradation be stored at -20°C until needed. citrate, pH=7.0) and 0.7% 2-mercaptoethanol. After vigorous vortexing the sample

#### Example 3: Reverse-transcriptase polymerase chain reaction

Half of total RNA was denatured for 5 min at 70°C, quickly chilled on ice and was reverse transcribed using random hexamer primers (Boehringer Mannh im, Mannheim, Germany). The synthesis was carried out with a First-strand cDNA

synthesis kit (Gibco, Eggenstein, Germany), including Superscript II (Gibco) in a final volume of 10  $\mu$ I containing 50 mM Tris (pH=8.3), 75 mM Kcl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM total dNTP, 1.6  $\mu$ g of the random primers and 100 units Superscript II. After the addition of RNA, the samples were incubated at 40°C for 1 hour and subsequently diluted with 10  $\mu$ I HPLC-water.

PCR-reaction mixture (10 µl) was composed of 1 µl cDNA, 1 µl of 10xPCR buffer (100 mM Tris, pH=8.3, 500 mM KCl, 10 mM MgCl<sub>2</sub>), 40 µM dNTP, 0.4 µM of each of the two primers, 5 µg BSA (Boehringer Mannheim, Mannheim, Germany). 0.6 units of Tag DNA-Polymerase (Boehringer Mannheim, Mannheim, Germany) and overlaid with 12.5 µl of mineral oil. The primers for the MAGE genes were designed from previously published sequences (Tab. 1, Fig.1), and were selected to maximize mismatches between the different MAGE-sequences particularly in the 3'-region in order to avoid cross-amplification. Specific PCR assays were possible for MAGE genes 1,2,4 and 12 since the percentage of identity of these genes varies between 64 to 85 %. In contrast, the sequence of Mage-6 was found to be 99% identical to that of Mage-3. Therefore our primers for Mage-3/6 hybridize to both genes and PCR can detect Mage-3 and/or Mage-6 expression. Short primers in the range of 19-22 bases and annealing temperatures of about 60°C were most efficient. Also junction primers spanning over two exons showed surprising results in efficiency. As published recently, we applied primers for PSA (Tab. 1) on exons 2 and 3 that span a 1.6-kilobase intron and amplify three different PSA cDNAs. To confirm the presence of cDNA template in each sample, primers specific for p53 (kindly provided by Thomas Blankenstein, München, Tab. 1) were used to coamplify this ubiquitously expressed "housekeeping" gene. Oligonucleotide primers were synthesized and purified at Genset, Paris. For the external fragment amplification of the MAGE PCR assays following cycling profile was used: denaturation at 94°C for 6 min, annealing at 60°C for 30 sec and extension at 72°C for 2 min for the first cycle; denaturation at 93°C for 40 sec, annealing at 60°C for 30 sec and extension at 72°C for 20 sec for 14 cycles; denaturation at 93°C for 40 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec for 50 cycles; terminal xtension at 72°C for 2 min and cooling at

of the nested PCR assay was 100%. of a malignant tumor cell line mixed with 1 ml normal whole blood. Reproducibility approach, the RT-PCR assays for the different MAGE genes could detect 10 cells amplification products obtained was monitored with DNA sequencing. Using this Germany) was used as a reference marker for all assays. The specificity of the bromide staining under UV-light. A 1 kb DNA ladder (Gibco BRL, Eggenstein, on 1.8% agarose by gel electrophoresis and by direct visualization after ethidium preparation and RT-PCR were performed routinely. PCR products were analyzed contaminations. Negative control reactions (mock reactions) for the RNA amplitication were performed in different rooms to avoid potential DNA crosslaminar air-flow bench with filtered pipette tips, external and nested fragment using plate control. Importantly, all pipetting was performed on ice under a was performed on a Hybaid Thermal Reactor (Biometra, Göttingen, Germany) 64°C and 7 cycles with 57°C, continuing 50 cycles with 57°C. The amplification and separating the 14 cycles into 7 cycles with and annealing-temperature of min. The cycling conditions for PSA varied by using conventional one-stage PCR 72°C (30 sec) with a final extension at 72°C for 2 min and cooling at 30°c for 1 amphitication thirty more cycles were run at 93°C (40 sec), 58°C (30 sec) and tube containing the PCR mixture described above. For the nested fragment 30°C for 1 min. One microliter of the reaction was then transferred into a second

#### Example 4: Immunocytochemistry

Germany; these as well as all other antibodies used in the experimental part of and later obtained from Dr. H. Bodenmüller, Boehringer Mannheim, Tutzing, antibodies CK2 (kindly provided by Dr. M. Osborn, Max Planck Institut Göttingen, minutes (5x10° cells per slide). BM specimens were stained with monoclonal for 30 minutes, interface cells were cytocentrifuged onto glass slides at 150g for 5 centritugation through Ficoll-Hypaque (Pharmacia, Freiburg, Germany) at 400g cells (MMC) from the remaining aspirate were isolated by density gradient Affer removal of 1 ml of whole BM and blood for PCR analyses, mononuclear

the application were prepared according to conventional procedures. The specific antibodies used here are not essential for carrying out the invention) and A-45-B/B3 (kindly provided by Micromet GmbH, Martinsried, Germany), which detect common epitopes on cytokeratin components. The antibody reactions were developed with the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique combined with the Neufuchsin method for visualizing antibody binding. Briefly, after incubation with the primary antibody, a polyvalent rabbit anti-mouse lg antiserum (Z259, Dako, Hamburg, Germany), and performed complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase antibodies (D651, Dako, Hamburg, Germany) were used at the dilutions recommended by the manufacturer (Dakopotts, Hamburg, Germany). For each BM aspirate, two slides containing a total of 106 MNC were evaluated

# Example 5: Expression of MAGE genes in normal bone marrow and blood versus other markers

RNA was extracted from BM of control patients and subjected to RT-PCR amplification using primers from several histogenetic differentiation markers and tumor-associated antigens. (Tab. 2). After 50 cycles of amplification, product of all these candidate genes known to be expressed in malignancies was seen except PSA and MAGE genes. Nevertheless, PSA expression is restricted to malignancies of the prostate and seems to be downregulated in a fraction of disseminated tumor cells. In contrast, MAGE genes are ubiquitously expressed in malignancies and no amplification product after sensitive nested PCR was seen in any of the analyzed 20 BM aspirates and 20 peripheral blood samples. This result was confirmed when analyzing bone marrow aspirates of 27 healthy persons. None of said aspirates from the allogenic bone marrow donors expressed the MAGE genes, see Table 5.

The highly restricted expression pattern of tumor antigens in contrast to tumorassociated molecules strongly supports that MAGE genes may be interesting candidates for PCR-based detection of minimal residual disease.

# Example 6: Expression of MAGE genes in bone marrow samples of patients with different types of tumors

.41 Gelelectrophoresis of Mage PCR products is shown in examples in Figures 5 to origin. Results and patient characteristics are listed in Table 4a-c and Table 7. blood samples from patients suffering from malignancies of different histological hematopoietic tissue encouraged us to evaluate BM aspirates and peripheral MAGE genes together with the complete absence of expression in normal showed MAGE-2 and -4 expression. This heterogenous expression pattern of the completely negative. Increasing the sensitivity by performing 65 cycles A498 therefore scored positive in the PCR. Only one renal cell carcinoma (A498) was with 35 cycles, 27 of 28 cell lines exhibited expression of at least one MAGE and hematopoietic and neuroectodermal origin (Tab. 3). Performing one-stage PCR RT-PCR assay in vitro and screened 28 cell lines of epithelial, mesenchymal, as shown for prostate cancer in Table 6, we initially evaluated our multimarker show a heterogenous expression pattern for different MAGE genes (1,2,4,6,1,6), disseminated tumor cells of different histological origin. Since primary tumors The objective of the study was to establish a PCR assay for detection of

### Prostate cancer

First, BM aspirates were obtained from both sites of the iliac creat of patients with prostate cancer (Tab. 4a, Tab. 7). Out of the 30 patients transcripts of MAGE-1 were observed in 12 patients (40%), MAGE-2 in 3 pat. (17%), MAGE-3/6 in 5 pat. (17%), MAGE-4 in 6 pat. (20%) and MAGE-12 in 5 pat. (17%). The overall pat. (17%), MAGE-4 in 6 pat. (20%) and MAGE gene in one patient was 18 (60%). Interestingly, only two patients showed concordant expression pattern of MAGE genes in both aspiration sites, whil 16 patients displayed heterogenous genes in both aspiration sites, whil 16 patients displayed heterogenous

expression. There was no correlation between the primary tumor stage, the histopathological grading or the mean PSA concentration in serum and the MAGE-positivity in the PCR-assay.

#### Lung cancer

The second epithelial tumor entity we were interested in was non-small and small lung cancer (Tab.4b). We analyzed single-sided BM aspirates of 34 carcinoma patients with positive results for MAGE-1 in 5 asp. (15%), MAGE-2 in 4 asp. (12%), MAGE-3/6 in 3 asp.(9%), MAGE-4 in 3 asp. (9%) and MAGE-12 in 4 asp.(12%). At least one MAGE expression was found in 12 asp. (35%). MAGE-PCR displayed positive signals in 7 of 19 BM aspirates of patients with squamous cell—carcinoma and 3 of 10 analyzed BM aspirates of patients with adenocarcinoma.

#### Sarcoma

Since mesenchymal tumor cell lines express different MAGE genes, we analyzed single-sided BM aspirates of 6 sarcoma patients staged as M1 using the sensitive MAGE PCR assays (Tab. 4c). 5 samples showed at least expression of one MAGE gene, while MAGE 1,2,4,12 were positive in two tumor samples, respectively, and MAGE-3/6 was positive in one sample. Transcripts of all MAGE genes were found in BM aspirates of patient T.C., suffering from rhabdomyosarcoma with infiltrating bone metastases.

# Example 7: Correlation of MAGE expression with other tumor cell dissemination markers

All BM aspirates of patients with malignancies of the lung and prostate were evaluated by both the MAGE RT-PCR and the well established immunocytochemical assay using mAb against cytokeratins. Expression of at least one MAGE gene and CK-status of 34 analyzed lung carcinoma samples is displayed in Fig.2. 12 aspirates showed positive results in MAGE PCR assay, while 8 aspirates rev aled CK positive cells. 6 aspirat s were found to be concordant positive and were all taken from patients with squamous cell

carcinoma. MAGE PCR showed positive results in 3 of 10 patients with adenocarcinoma, while immunocytochemistry exhibited no CK-positive cells in this bistolesical authors.

this histological subtype. CK-immunocytochemistry and a third assay: a sensitive RT-PCR for PSA. Fig.3 shows the results of PCR assays for MAGE and PSA, and of CK-immunocytochemistry. As mentioned above, out of 30 patients with prostate carcinoma 18 patients showed expression for at least one MAGE gene. PSA expression was observed in 8 patients, while CK-immunostained cells were found in 9 patients. Interestingly, all PSA positive results of the MAGE PCR assay. Comparatively, CK-immunostained cells were found patients. Moreover, seven PSA- and CK-negative patients revealed MAGE PCR assay. Comparatively, all PSA- and CK-negative patients revealed MAGE positive comparatively, and confirmed by positive results of the MAGE PCR assay.

# samples 8: Expression in blood samples compared to bone marrow

Most of recently published PCR assays claim to detect circulating tumor cells and predict high sensitivity; nevertheless the results of the analyses of PB samples obtained with CK-immunocytochemistry are disappointing. We analyzed 18 blood samples of patients with prostate carcinoma (n=12) and sarcoma (n=6), that were taken in parallel to the BM aspiration. As listed in Table 4a, MAGE-1 and MAGE-3/6 transcripts were found in one patient, while MAGE-4 expression could be observed in two patients with prostate cancer. Interestingly, two patients with PCR assay was completely negative for all analyzed PB samples. The obviously PCR assay was completely negative for all analyzed PB samples. The obviously lower sensitivity of the assay using peripheral blood was confirmed by analyzing patients with sarcoma (Tab. 4c). Only two patients revealed MAGE-2 and 3/6 expression, while the expression of the different MAGE genes in BM aspirates expression, while the expression of the different MAGE genes in BM aspirates could not be reproduced. Further, we analyzed 12 pati nts with advanced and

metastatic malignant melanoma using the sensitive MAGE RT-PCR assay. Expression of at least one MAGE gene was observed in only 5 patients with positive results for MAGE-1,2,3/6 and 12 in two samples and MAGE-4 in one sample. The failure to detect circulating cells in patients with localized or even advanced disease seems to indicate the bone marrow as preferential indicator compartment for detecting disseminated tumor cells.

# Example 9: Different expression of MAGE-genes in the primary tumor of prostate cancer patients

Total RNA was prepared primary tumors according to Chomczynski, Analytical Biochemistry 162 (1987) 156-159. Subsequently cDNA was synthesized as described in Example 6. As shown in Table 6, 7 of 20 tumors were positive for MAGE-1, 6 of 20 tumors for MAGE 2 and -12, respectively, 10 of 20 tumors for MAGE 3/6 and 4 of 20 for MAGE-4; overall, 14 of 20 tumors (70%) were positive for at least one MAGE gene product.

# Example 10: Different expression of MAGE-genes in the primary tumor and in the bone marrow of the same patient as determined by the method of the invention

Total RNA was prepared from the primary tumor of sarcoma patient H. M. (see also Tab. 4c) according to Chomczynski, Analytical Biochemistry 162 (1987) 156-159. Subsequently cDNA was synthesized as described in Example 3 followed by a one-step PCR with 35 cycles for MAGE 1, 2, 3/6, 4 and 12 as described in Example 6.

MAGE-analysis of a bone marrow sample of patient H. M. was carried out according to the method of the invention. Analysis of the primary tumor (results are shown in Figure 15) revealed predominant expression of MAGE-1 and only weak and inconsistent expression of other MAGE-genes including MAGE-4 and 12. Analysis of the bone marrow, however, only revealed the expression of Mage-

4 and -12, but no expression at all of MAGE-1 (Tab. 4c). Thus, the MAGE-1 expression pattern of systemically disseminated tumor cells does not necessarily match with that of the corresponding primary tumor. Therefore, therapeutic approaches according to claim 5, to eliminate systemically disseminated tumor cells thus preventing future distant metastasis should be preferably based on the analysis of the MAGE-expression pattern of said cells in an appropriate indicator tissue like bone marrow, rather than on analysis of the MAGE-expression pattern of the primary tumor.

### Example 11: Risk analysis of prostate cancer patients

analyzed, had progressive disease at the time of analysis as d fined by an prostat clomy or radiation (results are shown in Fig. 4a). The remaining 8 patients neoadjuvant therapy of their primary tumor prior to curatively intended of the 30 patients, at the time of first diagnosis of prostate cancer or during data were exlucively taken from Table 7). MAGE-expression was analysed in 22 positive for both aspiration sites. For the risk analysis of prostate cancer patients SC.FR. and LE.DI., respectively; in addition, patient JU.HA. turned out to be CKleft and negative results for the right bone marrow aspiration samples of patients reevaluation of the CK-immunocytochemistry data revealed positive results for the the time of first diagnosis of prostate cancer as given in Tab. 7. Finally, bone marrow aspiration, and therefore not identical with the initial PSA values at grading. Furthermore, the PSA values given in Tab. 4a are those at the time of data, there are some discrepancies to Table 7 with regard to tumor staging and listed as in Table 4a. However, as Table 4a contains more preliminary clinical listed in Table 7 and 4a (In Table 7 the same 30 prostate cancer patients are from the right and left iliac creat, respectively. Clinical data of the patients are -3/6, -4 or -12 was detectable in at least one of two bone marrow aspirates taken regarded MAGE-positive when at least one specific PCR-product for MAGE-1, -2, by bone marrow analysis according to the method of the invention. Patients were In 18 of 30 prostate cancer patients disseminated tumor cells could be detected

increasing blood concentration of PSA or by a local relapse; none of these patients, however, had clinical signs of distant metastasis at the time of MAGEanalysis. As it is well established in the art, these patients have a very high risk of future distant metastasis (Zietman, Cancer Supplement 71 (1993), 959-969; Fuks. Int. J. Radiation Oncology Biol. Phys. 21 (1991), 537-547; Pound, Urol. Clin. North Am 24 (1997), 395-406); thus it would be expected that most of them have already undergone systemic dissemination of cancer cells at the time of MAGEanalysis, when clinical evident distant metastasis has not yet developed but is likely to grow out of these disseminated single tumor cells in the future. Indeed, 7 of these 8 patients (87,5%) highly at risk of distant metastasis proved to be MAGE-positive. In contrast, only 11 of 22 patients (50,0%) analyzed in temporal association with their primary tumor, and thus representing both high and low risk patients according to future distant metastasis, were found to be MAGE-positive. Interestingly, only 22,7% of the patients in the latter group (5/22) were found to be MAGE-positive for both aspiration sites, compared to 50% in the high risk group (4/8). These data indicate, that detectable MAGE-expression in at least one bone marrow sample is associated with a high risk of future distant metastasis most probably due to the presence of systemically disseminated tumor cells. Thus, a subgroup of patients suffering from cancer and especially from prostate cancer being highly at risk of future distant metastasis may be identified by the method of the invention. This subgroup may be preferentially subjected to adjuvant cancer therapy according to claim 5 or any other adjuvant approach in order to prevent the outgrowth of distant metastases from systemically disseminated single cancer cells.

Table 1: Synthetic oligonucleotide primers for the nested RT-PCR amplification of the different MACE genes and the conventional RT-PCR amplification of PSA and pS3.

משפכשוכוני-3,	ฐวาวเวฐธวา- ่ 🤇	
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5'-೧೭೦೦೭೩ಲ್ಲಿ ಶ್ವಶ್ವ ಶ್ವರ್ಷ ಪ್ರಕ್ರಾತ್ರ	್ರೆ,-೧೯೦೦೮೩೯೭೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪೪	VIAGE-4
2,-ลีรเลียดเดเลี้สเตรลีลีสิตระ-3,	2,-สิรเสระเะเลิสเตรลีลีลีตรร-3,	
් - පුපුරුදු පුදු පුදු පුදු දි	් - පුඩ දුරු පුරු පුරු පුරු වැට දුරු - 3 °	MYCE-3\6
्र,-टर्ज्बर्वटप्रहेटडर्वाहेटाव्हेड्टाट-२,	2,-ಕೆಕಕೆಂತಕೆಕಕೆಕಕೆಕಕೆಕತೆಕರೆದಿದೆ-3,	
ೆ೧೩ಗಡ್ತಿಗೆ ಪ್ರಶ್ನತ್ತ ಪ್ರಕ್ಷಣ್ಣ ಪ್ರಕ್ಷಣ್ಣ ಪ್ರಕ್ಷಣ್ಣ ಪ್ರಕ್ಷಣ್ಣ ಪ್ರಕ್ಷಣ್ಣ ಪ್ರಕ್ಷಣ್ಣ ಪ್ರಕ್ಷಣ್ಣ ಪ್ರಕ್ಷಣ್ಣ ಪ್ರಕ್ಷಣ	`C-100ಶ್ರ101sgaagagaagaagine0-'Č	WYCE-5
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Table 2: Specificity of RT-PCR. Analyses of hematopoietic tissue from non-carcinoma control patients. Data are presented as positive bone marrow aspirates or PBL over total number of specimens assessed.

MARKER GENE	BONE	BLOOD
	MARROW	
1. Tumorassociated and histogenetic		
differentiation markers:		
CEA (Carcinoembryonic Antigen)	5/19	n.d.
erbB2	5/7	n.d.
erbB3	6/7	n.d.
EGP-40 (Epithelial Glycoprotein-40)	53/53	n.d.
Desmoplakin I	5/5	n.d.
Cytokeratin-18	<i>5/7</i>	n.d.
PIP (Prolactin-inducible Protein)	3/31	n.d.
PSM (Prostate-Specific Membrane	4/9	n.d.
Antigen)		, 
PSA (Prostate-Specific Antigen)	0/53	0/20
2. Tumorspecific Markers:	0/20	0/20
MAGE - 1	0/20	0/20
MAGE - 2	0/20	0/20
MAGE - 3/6	0/20	0/20
MAGE - 4	<i>0/</i> 20	0/20
MAGE - 12	0/20	0/20

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Table 3: Expression of the different MAGE genes by malignant tumor cell lines evaluated by specific RT-PCR amplification. Different levels of expression as deduced by band intensity of PCR products are represented by (+) or  $\div$ . Absence of PCR product is indicated by -.

+	(+)	+	+	+	K 562 (erythroleukemia)
<del>                                     </del>		-	-		RAJI (Burkitt-lymphoma)
+	<u> </u>	+	+	+	(amodamil tishuf) 11 A g
	+	+		+	. So Clymphoma) 759 U
(+)	+	+	+	+	LB-23 SAR (fibrosarcoma)
+	+	<del>                                     </del>	T -	T	HT 1080 (fibrosarcoma)
					nigiro lamrabozam
<u></u>			( )		2. Tumors derived of
+	+	+	(+)	+	MX-2 MEL (melanoma)
+	(+)		+	+	A 375 (melanoma)
+	- 9	+	+	+	MEL-MEI (melanoma)
+	+	+	+	+	MEL-JUSO (melanoma)
+	<u> </u>	-	+	+	U 138 (glioblastoma)
+	•	+	-	+	(smotssidoilg) SVI A
	1				neuroektodermal origin
_	l				IB. Tumors derived of
					(epidermoidcarcinoma)
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+	+	+	+ .	+	(gaul) 724 A
+	-	+	+	+	KATO (stomach)
+	(+)	-	+	+	PANC-TU (pancreas)
•	-	-	-	+	HEP G 2 (hepar)
+	-	+	+	+	НЕЬ В 3 (уєбяс)
-	-	-	-	+	CaKi I (kidney)
•	-	-	-	-	(Kidney) 849 A
÷	-	-	-	-	SW 480 (colorectal)
+	+	+	+	+	LS 180 (colorectal)
+	(+)	+	+	+	HT 29 (colorectal)
(+)	-	+	-	+	SkCo (colorectal)
+	-	+	-	÷	LNCaP (prostate)
+	-	+	+	-	MDA-MB (breast)
+	-	+	•	+	SkBr 3 (breast)
÷	+	+	+	+	BT 20 (breast)
+	-	+	-	+	MCF-7 (breast)
					nigiro
					ekto-/endodermal
					IA. Tumors derived of
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Tab.4: Clinical data and results (MAGE and PSA RT-PCR, CK-immunocytochemistry) on patients with a) prostate cancer

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TUMOR SIZE		C / 13.14		C/.13.14		1.I. / V		D / 1'4		C / 13.14		. B / T1112		C / 13.14		1.L/Y	-	1.1. / V		C / 1314		C / 13T4		C / 113114		C / 1314	
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Tab.4: Clinical data and results (MAGE and PSA RT-PCR, CK-immunocytochemistry) on patients with b) lung cancer

					<u> </u>									31		_	<del></del> -	<del>,</del>	-	_		_		_	_	_		_	_	τ-	_
	CK-IMMUNO	+	÷	÷	+	+		•	+	-		•	•	÷	+	-	٠					,	,	1			•	-			
	MAGE-12	+				+	÷	•	•	•	•		ż	•	•		•	٠		•			•	1		-	÷		•		•
W ANALYSES	MAGE-4	+	,		٠	-	-	÷	•	+		·			•					1	,					•			•	•	
HONE MARROW ANALYSES	MAGE-3/6	+	,		-			÷	•	,	•		٠		+			,					•	•			1	•		•	
	MAGE-2	•	ı		+		•	•	+	•	•	+		+	•	1	,			•		•	•	•	•	-	•	•	-	•	•
	MAGE-1	+	1	•	-	•	+		•	1	•	•		r	+	•	•	•	•	•	•	•	e	•	+		+	•	•	•	•
STACING		TZNOMO	OMINZI.	IZNIM0	TANOMO	IZNZM0	IZNIM0	TZNZMO	TZNIMO.	T4N2M0	TINOMO	TAN3MO	TINOMO	OMONZI.	TINIMO	TZNIMO	TZNOMO	TZNOMO	TAN3MO	OMINZI.	TZN I MO	TOMOMO	S 12N2M0	TZNZMO	TINIMO	UZNIMO	OMONZL	IZNZM0	OMZNZI.	TANIMO	TZNOMO
HISTOLOGY		SOUAMOUS	NHOLSVIII	SOUAMOUS	SOUAMOUS	SOUAMOUS	SCLC	ADENO	SOUAMOUS	ADIENO	ADENO	ADENO	ADENO	SOUAMOUS	SOUAMOUS	SOUAMOUS	SOUAMOUS	ADENO	SUOMAUDS	ADENO	SUOMAUOS	SARCOMA	VDENO	ONEGV	SOUMAUS	SOUMAUS	LARGIS-CISLL	SOUAMOUS	SOUAMOUS	SOUAMOUS	SUOMANUS
PATIENT		VO.KL.	AR-JO.	WE.AL.	RO.AD.	GA.AB.	F1.R1.	RE.LU.	1113.10.	KU.LU.	KL.JU.	LÖ.MA.	IIA.AN.	CR.GO.	DE.IV.	ие. ги.	MA.RO.	114.111.	110.FR.	MA.10.	LA.BR.	IN.FR.	1.Α.Α1	SC.10.	21.10.	KR.WI.	NI.OS	80.10.	NR.WI.	BA.MA.	BR.FE.

SC.JO.	PO.MA.	IV.MA.	MA.RI.
ADENO	LARGE-CELL	SOMVNOS	SONWOOS
OWINZI.	T2NIM0	TZNOMO	T3N2M0
	•	•	•
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•	•	-	•
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	•	•	•
•	•	•	-

Tab. 4b (continue)

Tab.4: Clinical data and results of MAGE RT-PCR on patients with c) sarcoma

			-						
PATIENT	IIISTOLOGY	LOCALIZATION OF M-STAGE PRIMARY TUMOR	M-STAGE		HONE	HONE MARROW ANALYSES	LYSES		PII ANALYSES
				MAGE-1	MAGE-2	MAGE: 3/6 MAGE: 4 MAGE: 13	MACELA	MAGE: 12	
G.	SYNOVIAL SARCOMA	RIGHT FEMUR	Ξ					TI-STATE OF	
R.T.	LEIOMYOSARCOMA	RIGHT FEMUR	ž	•					
R.R.	BPITHELOID SARCOMA	PARAVERITEBRAL	Σ						•
	17100a 1800x10i3 1		,				_	•	•
زد	LEIUM TUSAIKCUMA	PELVIS MINOR	Ξ	+	•			•	
D.K.	EWING'S SARCOMA	MULTILOCULAR	ω		+	,			
H.M.	UNDIFFERENTIATED	CARDIAC STOMACIL	οM				4	1	•
	SARCOMA							<del>-</del>	•
T. L.	RHAIIDOMYOSARCOMA	PELVIS MINOR	Ξ	+	+	+	7	T	

Tabl 5: Analys s of bone marrow aspirates of 27 healthy p reons (= allogeneic bone marrow donors). Data are presented as negative and positive results in RT-PCR of total number of specimens assessed.

WAGE-12	0	72
WYGE-4	0	27
WAGE-3/6	0	72
MAGE-2	0	72
MAGE-1	0	72
	PCR-pos.	PCR-neg.
WARKER GENE	BONE MAR	MOS

**Tabl 6:** Expression of the different MAGE genes by primary tumors of 20 patients with prostate cancer. Evaluation was obtained by specific RT-PCR amplification of native malignant prostatic tissue. Data are presented as negative and positive results in RT-PCR of total number of specimens assessed.

MARKER GENE	PROSTATI	ETUMOR
	PCR-pos.	PCR-neg.
MAGE-1	7	13
MAGE-2	6	14
MAGE-3/6	10	10
MAGE-4	4	16
MAGE-12	6	14
at least one MAGE	14	6

Clinical Time of Stage   Stage   Histology   CK   Stage   Jirst   Grade   C   Le																
	Patient	Clinical	Time of	Stage/ Grade	Histology/ Grade	CX	PSA-	PSA-	Bone I	Marrow MA	PCR-A	nalyses 1es	gnisu	Time of aspiration	Treat	Follow-up
C 121994 TNAMA/P Adeno/2 +/- 1118 -/- +/- +////////-		•	diagnosis				Level									
C 121994 T,N,M,P Adeno/2 ++ 11.8 ++ ++ ++ ++ ++ ++ ++ ++ ++ ++ ++ ++ ++	•		. 1				(hg/l)		I	2	3/6	4	12			
C 121994 T,N,MA,P Adeno/2 + 11.8 + + + + + + + + +-								Ur	Ur	Ur	Ur	Ur	Ur			
C 6/1996 TNA%/P Adeno/2	SC.AU.	ဂ	12/1994	T,NoMo/p	Adeno/2	<b>‡</b>	11.8	-/-	-/+	-/+	-/-	<i>-</i> /-	-/-	0	A	BCR 5/96
B 9/1995 T <sub>1</sub> N <sub>1</sub> M <sub>2</sub> /p Adeno/2 -+ 5.4 ++ ++ ++ ++ ++ D 12/1994 T <sub>1</sub> N <sub>1</sub> M <sub>2</sub> /p Adeno/3 -+ 19.4 -+ ++ -+ ++ -+ ++ ++ ++ ++ ++ ++ ++ ++	SE.NI.	ဂ	6/1996	T,NoMo/p	Adeno / 2	<u>+</u>	11.6	-/+	<b>*</b>	+	<u>+</u>	+	+	×	>	NR 11/97
D 12/1994 T,N,M,/b Adeno/3 -/+ 1444 -/+ -/////////-	BA.HE.	₩	9/1995	T,N,M,/p	Adeno / 2	<b>‡</b>	5.4	<i>+</i>	<del>+/+</del>	-/-	<del>+</del> /+	‡	+	×	>	NR 1/98
C 6/1994 T,N,Ma/P Ademo/1 +/- 9.9 -/+ -////- B na. T,N,Ma/P Ademo/0 +/- 15.1 -/+ +/////////-	RI.ER.	ם	12/1994	T,N,M,/6	Adeno / 3	<b>‡</b>	144.4	<u>-</u> /+	+	-/-	+/-	+	<u>+</u>	×	ם	BCR 96 DM 97
B n.a. T <sub>0</sub> N <sub>0</sub> M <sub>0</sub> /p Ademo/0 ++- 15.1 -++ +++++- ++- B 12/1991 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Ademo/3 -+- n.a++++++++++	SC.FR.	ဂ	6/1994	T2NoMo/p	Adeno / 1	<u></u>	9.9	<del>'</del> +	<u>+</u>	+	<u>+</u>	<b>+</b>	+	0	>	BCR 11/95
B 12/1991 T,N,M,/b Adeno/3 -/- n.a//////////	LE.DI.	₩	n.a.	T,N,M,/p	Adeno / 0	<b></b>	15.1	+	+	+	<u>+</u>	<u>+</u>	+	×	>	n.a.
A 10/1995 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno / 2 -/- 9.51 -////////- C 1992 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno / 3 -/- n.a/- +/////////-	WO.JA.	₩	12/1991	T,N,M,/b	Adeno/3	<u></u>	n.a.	-/-	<u></u>	<u>+</u>	+/-	<u>+</u>	<u>+</u>	0	ם	BCR 5/96
C 1992 TJN,M6/P Adeno/3	BE.WA.	>	10/1995	T2NoMo/p	Adeno/2	<u>+</u>	9.51	-/-	<b>/</b> -	<u>+</u>	<i>\</i>	<u>+</u>	<u>+</u>	×	>	NR 3/97
C 7/1994 TJN,M/P Adeno/3 -/- n.a//////////	GE.CH.	ဂ	1992	T,N,M <sub>0</sub> /p	Adeno / 3	<u>+</u>	n.a.	<b>;</b>	<b>+</b> ;-	<i>-</i> -	<b>;</b>	+	<u>+</u>	0	txi	LR 96, DM 97
C 10/1992 T <sub>1</sub> N <sub>2</sub> M <sub>6</sub> /p Adeno/2 -/- n.a. +/+ +//////- C 7/1996 T <sub>2</sub> N <sub>2</sub> M <sub>6</sub> /p Adeno/2 -/- 134 -//////////	HE.RU.	ဂ	7/1994	T,NoMo/p	Adeno / 3	<b>+</b>	n.a.	<i>-</i> -	<b>-</b> /-	<b>-</b> -	<b>+</b>	<u></u>	+	0	ଦ	<b>BCR 6/96</b>
C 7/1996 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 -/- 134 -///////- B 7/1996 T <sub>2</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 +/+ 5.25 -//////////	KN.DI.	ი	10/1992	T,NoMo/p	Adeno / 2	<u>+</u>	п.а.	<b>‡</b>	<b>‡</b>	<i>-</i> -	<u>-</u> -	<u>+</u>	<u></u>	0	<b>A</b>	BCR + LR /96
C 7/1996 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 +/+ 5.25 -//////////	MEJO.	ဂ	7/1996	T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /b	~	<u>+</u>	134	<u>+</u>	<u>+</u>	<u>-</u> -	<u>+</u>	<u>}</u>	<u>+</u>	×	В	NR 1/98
B 7/1996 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 -/+ 8.2 -///////- C 1992 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 -/- 9.27 -//////////	JU.HA.	ဂ	7/1996	T,N,M,/p	$\overline{}$	#	5.25	<u></u>	<u>+</u>	<u>+</u>	+	<u>+</u>	<u>+</u>	×	>	NR 1/98
C 12/1995 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 -/- 9.27 -///////- A 10/1995 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 -/- n.a//////////	WE.HJ.	₩	7/1996	T2NoM Jb	_	<b>‡</b>	8.2	<u>-</u> -	<u>-</u>	<u> </u>	<u>+</u>	+	<del>'</del>	×	n.a.	n.a.
C 1992 T <sub>1</sub> N <sub>2</sub> M <sub>4</sub> /p Adeno/2 n.a A 10/1995 T <sub>2</sub> N <sub>2</sub> M <sub>4</sub> /p Adeno/0 15.7	PF.LO.	ი	12/1995	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub> /p	~	+	9.27	<u>+</u>	<u>+</u>	<u>+</u>	+	<u>+</u>	<u>+</u>	×	>	n.a.
A 10/1995 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/0 -/- 15.7 -//////////	SC.EU.	ი	1992	T,N <sub>0</sub> M <sub>0</sub> /p	_	+	n.a.	+	<del>-</del>	+	+	+	<u>+</u>	0	>	BCR + LR 96
C 4/1996 T <sub>1</sub> N <sub>2</sub> M <sub>4</sub> /p Adeno/2 -/- 0.777 -//////- A 3/1996 T <sub>1</sub> N <sub>3</sub> M <sub>4</sub> /p Adeno/2 -/- 9.14 -////////- A 5/1996 T <sub>1</sub> N <sub>4</sub> M <sub>4</sub> /p Adeno/2 -/- 9.14 -//////////	SC.GU.	>	10/1995	T,N,M,/p	•	+	15.7	<u>+</u>	<del>-</del>	<u>+</u>	<u>}</u>	<u>-</u> -	<i>-</i> /-	×	>	LR 11/97
A 3/1996 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 -/+ 41.4 -//+ -///- A 5/1996 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 -/- 9.14 -/////////- C 8/1995 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/3 -/- 56.3 +/+ +/////////-	KU.WI.	ი	4/1996	T2NoMo/p		<b>÷</b>	0.777	+	+	<u>+</u>	<u></u>	+	+	×	נבי	LR+DM 97
A 5/1996 T <sub>1</sub> N <sub>1</sub> M <sub>0</sub> /t Adeno/2 -/- 9.14 -///////- C 8/1995 T <sub>2</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/3 -/- 56.3 +/+ +///////- C 7/1996 T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/3 -/- 23.8 -//+ -/+ -/+ -/////- C 7/1996 T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 -/- 14.5 -/+ -/////////-	RI.FE.	>	3/1996	T,NoMo/p	Adeno / 2	<b>‡</b>	41.4	+	<b>;</b>	<u>+</u>	<u>+</u>	+	<i>+</i>	×	>	iBCR 1/98
C 8/1995 T <sub>1</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/3 56.3 +++ + ++ -++ C 7/1996 T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/3 23.8+ ++ ++ C 7/1996 T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 14.5 + +- +- +- C 1/1996 T <sub>2</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 13.0 +++ + +- +- +- C 5/1996 T <sub>2</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/0 20.4 C 3/1996 T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/1 20.4 C 3/1995 T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 5.31 B 5/1996 T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 15.2 C 5/1996 T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 15.2 C 5/1996 T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /p Adeno/2 15.2	ST.EB.	>	5/1996	T,N,Mo/t	Adeno / 2	<u>+</u>	9.14	+	<u>+</u>	<u>+</u>	<u>+</u>	<u>+</u>	<u></u>	×	റ	BCR 3/98
C 7/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/3 -/- 23.8 -//+ -///- C 7/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/2 -/- 14.5 -/+ -///- +/+ -/////-	SC.HO.	ი	8/1995	T,NoMo,Lp	_	+	56.3	<b>‡</b>	<del>+/-</del>	+	+	+	+	×	>	BCR 3/98
C 7/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/2 -/- 14.5 -/+ -///- +/+ C 1/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/2 -/- 13.0 +/+ +/+ -/////- C 5/1996 T <sub>5</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/0 -/- 20.4 -//////- C 3/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/1 -/- 20.4 -//////- C 7/1995 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/2 -/- 5.31 -/- +//////- B 5/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/2 -/- 14.0 -///////- C 5/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/2 -/- 15.2 -//////////	FE.KU.	ი	7/1996	T <sub>3</sub> N <sub>0</sub> M <sub>0</sub> /p	_	+	23.8	<u>+</u>	<b>;</b>	<b>;</b>	+	<u>+</u>	<u>-</u>	×	>	n.a.
C 1/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/2 -/- 13.0 +/+ +/+ -/////- C 5/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/0 -/- 20.4 -////////- C 3/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/1 -/- 4.2 -//////////	LU.VO.	ဂ	7/1996	T,NoMo/p	Adeno / 2	<u>+</u>	14.5	<b>;</b>	<del>-</del> /-	<u>+</u>	+	+	++	×	>	NR 10/97
C 5/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/0 -/- 20.4 -/////- C 3/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/1 -/- 4.2 -//////- C 7/1995 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/2 -/- 5.31 -/- +//////- B 5/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/2 -/- 31.5 -//////////	zo.jo.	ဂ	1/1996	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub> /p	Adeno / 2	<u>+</u>	13.0	<b>‡</b>	<b>‡</b>	<u>+</u>	<u>+</u>	+	<b>†</b>	×	>	iBCR 3/98
C 3/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno / 1 -/- 4.2 -/////- C 7/1995 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno / 2 -/- 5.31 -/- +//////- B 5/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno / 2 -/- 15.2 -//////////	MA.GU.	ဂ	5/1996	T,N,M,/p	Adeno / 0	<u>+</u>	20.4	<u>+</u>	+	<u>+</u>	+	<u>+</u>	<u>+</u>	×	<b>A</b>	NR 1/98
C 7/1995 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/2 -/- 5.31 -/- +////- B 5/1996 T <sub>3</sub> N <sub>6</sub> M <sub>6</sub> /p Adeno/2 -/- 31.5 -//////////	BA.GI.	ဂ	3/1996	T,N <sub>0</sub> M <sub>0</sub> /p	Adeno / 1	<u>+</u>	4.2	<u>+</u>	<u>+</u>	<u>-</u> -	<u>+</u>	+	<u>+</u>	×	₩	NR 5/97
C 8/1995 T <sub>1</sub> N <sub>2</sub> M <sub>2</sub> /p Adeno/2 -/- 14.0 -/////- B 5/1996 T <sub>1</sub> N <sub>2</sub> M <sub>2</sub> /p Adeno/2 -/- 31.5 -//////////	WLJA.	ဂ	7/1995	T2NoMo/p	Adeno / 2	<u>+</u>	5.31	<u>+</u>	<b>+</b> ,-	<i>-</i> -	+	+	<u>+</u>	•	<b>A</b>	BCR+LR 96
B 5/1996 T <sub>2</sub> N <sub>2</sub> M <sub>2</sub> /p Adeno/2 -/- 31.5 -/////////- C 5/1996 T <sub>2</sub> N <sub>2</sub> M <sub>2</sub> /p Adeno/2 -/- 15.2 -///////-	AK.HA.	ဂ	8/1995	T <sub>2</sub> N <sub>0</sub> M <sub>0</sub> /p	Adeno / 2	<u>+</u>	14.0	<i>+</i> -	<b>+</b>	-/-	<u>-</u> -	<u>+</u>	<u>+</u>	×	<b>A</b>	NR 11/97
C 5/1996 T.N.M./p Adeno/2 -/- 15.2 -////-	BLGU.	₩	5/1996	T,N,M,/p	Adeno / 2	<u>+</u>	31.5	<b>!</b> -	<b>-</b>	<u>+</u>	<u>-</u> -	<u></u>	<u></u>	×	٨	NR 12/97
	HI.ER.	ဂ	5/1996	T,N,M,/p	Adeno / 2	<del>-</del>	15.2	<b>+</b>	<u></u>	<u>+</u>	<u>-</u> -	+	+	×	Α	NR 2/98

#### Table 7-legend:

Clinical data of 30 patients with prostate cancer and the corresponding results of MAGE RT-PCR, PSA RT-PCR and CK-Immunocytochemistry as obtained by the analysis of bone marrow samples from the right and left iliac crest, respectively. Table lists: patients, clinical stage A-D, time of first diagnosis, histology (adenocarcinoma = adeno, neuroendocrine carcinoma = neuro) and corresponding grading (1=well differentiated, 2= moderately differentiated, 3= poorly differentiated), TNM-stage related to biopsy and lymphadenectomy (= b) or related to prostatectomy and lymphadenectomy (= p), PSA-level prior to therapy (= initial PSA), results of immunocytochemistry and RT-PCR, time of aspiration (0 = bone marrow aspiration at the time of progressive disease but without clinically evident distant metastasis, x = bone marrow aspiration at the time of first diagnosis of prostate cancer or during neoadjuvant therapy of the primary tumor prior to curatively intended prostatectomy or radiation), treatment modality (A = neoadjuvant antiandrogen therapy and subsequent prostatectomy, B = neoadjuvant antiandrogen therapy and subsequent radiation, C = transurethral resection, D = primary antiandrogen therapy, E = prostatectomy and adjuvant antiandrogen therapy, F = neoadjuvant antiandrogen therapy plus chemotherapy, and subsequent prostatectomy, G = prostatectomy), as well as time and result of last clinical follow-up ( NR = no relapse, BCR = biochemical relapse as defined by continuously PSA levels over prolonged periods of time, iBCR = initially increasing PSA level over a short period of time prior to end of follow-up, LR = local relapse, DM = clinically evident distant metastasis). n.a. = not available.

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(i) APPLICANT:

(E) CONNIEX: GERMANY

enon : STATE (d) (C) CIIX: Planegg

(iv) COMPUTER READABLE FORM:

(iii) NUMBER OF SEQUENCES: 20

(B) STREET: Am Klopferspitz 19

(E) BORLYT CODE (SIB): 85725

of disseminated tumor cells

(C) OBEKFLING SKSLEW: BC-DOS/WS-DOS (B) COMPUTER: IBM PC compatible (A) MEDIUM TYPE: Floppy disk

(A) DESCRIPTION: \desc = "oligonucleotide"

\desc = "oligonucleotide"

(D) SOFTWARE: Patentin Release #1.0, Version #1.30 (EPO)

(ii) TITLE OF INVENTION: Novel primers and methods for the detection

(ii) MOLECULE TYPE: other nucleic acid

(x;) SEGNENCE DESCRIBLION: SEG ID NO: I:

(ii) MOLECULE TYPE: other nucleic acid

(D) TOPOLOGY: linear (C) STRANDEDNESS: single (B) TYPE: nucleic acid (A) LENGTH: 21 base pairs (i) SEQUENCE CHARACTERISTICS:

(S) INFORMATION FOR SEQ ID NO: 2:

(iii) HYPOTHETICAL: YES

(A) DESCRIPTION:

(S) INEOKWYLION LOK SEÓ ID NO: I:

(D) TOPOLOGY: linear (C) SIKYNDEDNESS: single (B) TYPE: nucleic acid (A) LENGTH: 21 base pairs (1) SEQUENCE CHARACTERISTICS:

O AABBAABOOD BOTTBABATB

(A) NAME: Micromet GmbH

(I) GENEKAL INFORMATION:

SEQUENCE LISTING

- (iii) HYPOTHETICAL: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CAGGAGCTGG GCAATGAAGA C

21

- (2) INFORMATION FOR SEQ ID NO: 3:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 22 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CATTGAAGGA GAAGATCTGC CT

22

- (2) INFORMATION FOR SEQ ID NO: 4:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 22 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"
- (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GAGTAGAAGA GGAAGAAGCG GT

22

- (2) INFORMATION FOR SEQ ID NO: 5:
  - (i) SEQUENCE CHARACTERISTICS:

- (XI) SEGNENCE DESCRIBLION: SEG ID NO: 1:
  - (iii) HYPOTHETICAL: YES
- (ii) MOLECULE TYPE: other nucleic acid
  (A) DESCRIPTION: \desc = "oligonucleotide"

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- (D) TOPOLOGY: linear
- (C) STRANDEDNESS: single
  - (B) TYPE: nucleic acid
- (A) LENGTH: 21 base pairs
- (i) SEQUENCE CHARACTERISTICS:
- (S) INFORMATION FOR SEQ ID NO: 7:

50

#### AADDDDADTO DTDTDADTAD

- (xt) SEGUENCE DESCRIBLION: SEG ID NO: 6:
  - (iii) HYPOTHETICAL: YES
- - (D) TOPOLOGY: linear
  - (C) STRANDEDNESS: single
  - (B) TYPE: nucleic acid
  - (A) LENGTH: 20 base pairs
  - (i) SEQUENCE CHARACTERISTICS:
  - (S) INFORMATION FOR SEQ ID NO: 6:

18

#### BAAGCCGGC CAGGCTCG

- (x;) SEGUENCE DESCRIPTION: SEQ ID NO: 5:
  - (iii) HYPOTHETICAL: YES
- (A) DESCRIPTION:  $\ensuremath{\text{\degree}}$  desc = "oligonucleotide"
  - (ii) MOLECULE TYPE: other nucleic acid
    - (D) TOPOLOGY: linear
    - (C) STRANDEDNESS: single
      - (B) TYPE: nucleic acid
    - (A) LENGTH: 18 base pairs

CACCAAGGAG AAGATCTGCC T

- (2) INFORMATION FOR SEQ ID NO: 8:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 21 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TCCTCAGTAG TAGGAGCCTG T

21

- (2) INFORMATION FOR SEQ ID NO: 9:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 20 base pairs
      - (B) TYPE: nucleic acid
      - (C) STRANDEDNESS: single
      - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

- (iii) HYPOTHETICAL: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCCGTGAGGA GGCAAGGTTC

20

- (2) INFORMATION FOR SEQ ID NO: 10:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 22 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"

- (i) SEQUENCE CHARACTERISTICS:
- (S) INFORMATION FOR SEQ ID NO: 13:

20

CTGGGCAATG AAGACCCACA

(x;) SEĞNENCE DESCKIBLION: SEĞ ID NO: 15:

- (iii) HYPOTHETICAL: YES
- (ii) MOLECULE TYPE: other nucleic acid
  (ii) MOLECULE TYPE: other nucleic acid
  - (D) TOPOLOGY: linear
  - (C) STRANDEDNESS: single
  - (B) TYPE: nucleic acid
  - (A) LENGTH: 20 base pairs
  - (i) SEQUENCE CHARACTERISTICS:
  - (S) INLOWWATION LOW SEG ID NO: 13:

20

TAGAGTTCGG CCGAAGGAAC

- (x;) SEĞNENCE DESCEIBLION: SEĞ ID NO: II:
  - (iii) HYPOTHETICAL: YES
- - - (D) TOPOLOGY: linear
    - (C) SIRANDEDNESS: single
      - (B) TYPE: nucleic acid
    - (A) LENGTH: 20 base pairs
    - (i) SEQUENCE CHARACTERISTICS:
    - (S) INLORWATION FOR SEQ ID NO: 11:

22

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- (x;) SEGNENCE DESCRIBLION: SEG ID NO: 10:
  - (iii) HABOLHELICYF: AES

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "oligonucleotide"
- (iii) HYPOTHETICAL: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CATTGAAGGA GAAGATCTGC CT 22

- (2) INFORMATION FOR SEQ ID NO: 14:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 20 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CAGGCTTGCA GTGCTGACTC

. 20

- (2) INFORMATION FOR SEQ ID NO: 15:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 19 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
(A) DESCRIPTION: \desc = "oligonucleotide"
           (ii) MOLECULE TYPE: other nucleic acid
```

- (D) TOPOLOGY: Linear
- (C) STRANDEDNESS: single
  - (B) TYPE: nucleic acid
- (A) LENGTH: 21 base pairs
- (i) SEQUENCE CHARACTERISTICS:
- (S) INFORMATION FOR SEQ ID NO: 18:

CACCAAGGAG AAGATCTGCC T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

#### (iii) HYPOTHETICAL: YES

\qeac = "oligonucleotide" (A) DESCRIPTION: (ii) MOLECULE TYPE: other nucleic acid

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- (D) TOPOLOGY: linear
- (C) STRANDEDNESS: single
  - (B) TYPE: nucleic acid
- (A) LENGTH: 21 base pairs
- (i) SEQUENCE CHARACTERISTICS:
- (S) INFORMATION FOR SEQ ID NO: 17:

02

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(x;) SEĞNENCE DESCHIBLION: SEĞ ID NO: 10:

#### (iii) HYPOTHETICAL: YES

- (A) DESCRIPTION: \desc = "oligonucleotide"
  - (ii) MOLECULE TYPE: other nucleic acid
    - (D) TOPOLOGY: linear
    - (C) SIRANDEDNESS: single
    - (B) TYPE: nucleic acid
    - (A) LENGTH: 20 base pairs (i) SEQUENCE CHARACTERISTICS:
    - (S) INFORMATION FOR SEQ ID NO: 16:

6 I

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- (iii) HYPOTHETICAL: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

45

CAGGCTTGCA GTGCTGACTC T

- (2) INFORMATION FOR SEQ ID NO: 19:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 20 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear

  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TCCGTGAGGA GGCAAGGTTC 20

- (2) INFORMATION FOR SEQ ID NO: 20:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 19 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "oligonucleotide"
  - (iii) HYPOTHETICAL: YES
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GAGCCTGCGC ACCCACCAA

19

### **CLAIMS**

primers having the same 3' portion as one of the following groups **(i)** strand thereof, said primer being selected from the group of a MAGE tumor-specific antigen or a part thereof or to a complementary complementary to the messenger RNA transcribed from a gene encoding A primer specifically hybridizing to a nucleic acid molecule

of primers:

**(9)** 

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	(q)
(SEØ ID NO: 10)	5'-tccgtgaggaggcaaggttc-3' 5'-atcggattgactccagagagta-3'
(SEQ ID NO: 7) (SEQ ID NO: 8)	5'-caccaaggagagatctgcct-3' 5'-tcctcagtagtaggagcctgt-3'
(2EC) ID NO: 6)	5'-gaagccggcccaggctcg-3' 5'-gatgactctggtcaggcaa-3'
(2EC) ID NO: 4)	2,-ĉeĉĥeĉasĉaŝaŝascccc-3,
(SEQ ID NO:2)	5'-caggagctgggcaatgaagac-3'

2,-მმදෙදෙම් වැරිපුම් පිටිදු ද (SEQ ID NO: 15) (SEQ ID NO: 14) 5'-caggcttgcagtgctgactc-3' 5'-cattgaaggagaagatctgcct-3' (SEQ ID NO: 13) (SEQ ID NO: 12) 5'-ctgggcaatgaagacccaca-3' 2,-tagagttcggccgaaggaac-3' (SEQ ID NO: 11)

2,-csddcttdcsdtdctdsctct-3, (SEQ ID NO: 18) (SEQ ID NO: 17) 5'-caccaaggagaagatctgcct-3' (SEQ ID NO: 16) 2,-datgactctggtcagggcaa-3,

2,-dagcctgcgcsccacca-3, 5'-tccgtgaggaggcaaggttc-3'

(SEQ ID NO: 20)

(SEQ ID NO: 19)

MENOTERO ON THE STOOMS IN

;and

- (ii) primers that overlap with a primer sequence depicted in (a) or (b).
- The primer according to claim 1 which is a primer depicted in either group
   (a) or group (b).
- 3. A diagnostic composition comprising at least 4 primers according to claim 1 or 2 wherein said at least four primers hybridize pair-wise to strands of opposite orientation of at least two different nucleic acid molecules wherein strands of one orientation are complementary to the mRNA transcribed from the genes of at least two different MAGE tumor-specific antigens.
- 4. A method of detecting disseminated tumor cells indicative of a cancerous condition in a patient comprising:
  - (a) carrying out PCR on cDNA obtained from mRNA from one or more patient samples using at least two primers according to claim 1 or 2 hybridizing pair-wise to strands of opposite orientation of at least one cDNA molecule wherein strands of one orientation are complementary to the mRNA transcibed from the genes of at least one MAGE tumor-specific antigen; and
  - (b) detecting one or more PCR products.
- 5. A method of preparing a tumor adjuvant vaccine comprising the following steps:
  - (a) carrying out PCR on cDNA obtained from mRNA from one or more patient samples using at least four primers according to claim 1 or 2 hybridizing pair-wise to strands of opposite orientation of at least two different nucleic acid molecules wherein strands of one orientation are complementary to the messenger RNA transcribed

from the genes of at least two different MAGE tumor-specific antigens;

- (b) detecting resulting PCR products; and
- using at least one MAGE-gene product, expression of said MAGE-gene being detected as a result of step (b) for preparing an adjuvent furnor vaccine based on MAGE-antigen-derived peptides resembling MHC-restricted T-cell-epitopes, whole MAGE-antigens or fragments thereof, MAGE-transfected host cells, DNA vaccinating strategies using MAGE-encoding nucleotide sequences or any other immunization method based on MAGE-gene products or their coding nucleotide sequences.
- 6. The method according to claim 4 or 5 wherein said cDNA is obtained by:
  (a) preparing mRNA from one or more samples removed from said
- patient; and (b) reverse transcribing said mRNA.

nested PCR.

- 7. The method according to any one of claims 4 to 6 wherein said PCR is
- The method according to any one of claims 4 to 7, wherein said primers hybridize to 2 to 6 different nucleic acid molecules complementary to the mRNA transcribed from the genes of MAGE fumor-specific antigens and the complementary strands thereof.
- 9. The method according to any one of claims 4 to 8 wherein said MAGE tumor-specific antigens are the MAGE -1, -2, -3, -4, -6, or -12 tumor-specific antigens.
- 10. The method according to any one of claims 4 to 9 wherein said samples are removed from two diff rent aspiration sites.

- 11. The method according to any one of claims 4 to 10 wherein said samples are bone marrow aspirates.
- 12. The method according to any one of claims 4 to 11, wherein said mRNA preparation comprises the following steps:
  - (a) immediately lysing the sample in buffer essentially completely avoiding RNA-degradation; and
  - (b) optionally centrifuging the mRNA obtained in the lysate through a cushion of an RNAse-inhibiting agent, preferably cesium trifluoroacetate.
- 13. The method according to any one of claims 4 to 12 wherein said cancerous condition is related to prostate cancer, non-small or small lung cancer, sarcoma, malignant melanoma, breast cancer or colorectal cancer.

external as well as internal amplification is indicated. Arrows indicate the position of the oligonucleotides used for RT-PCR analyses. The expected size of the PCR products in the Fig.1: Location of the specific oligonucleotide primers in the MAGE gene sequences. Grew boxes show the different exons.

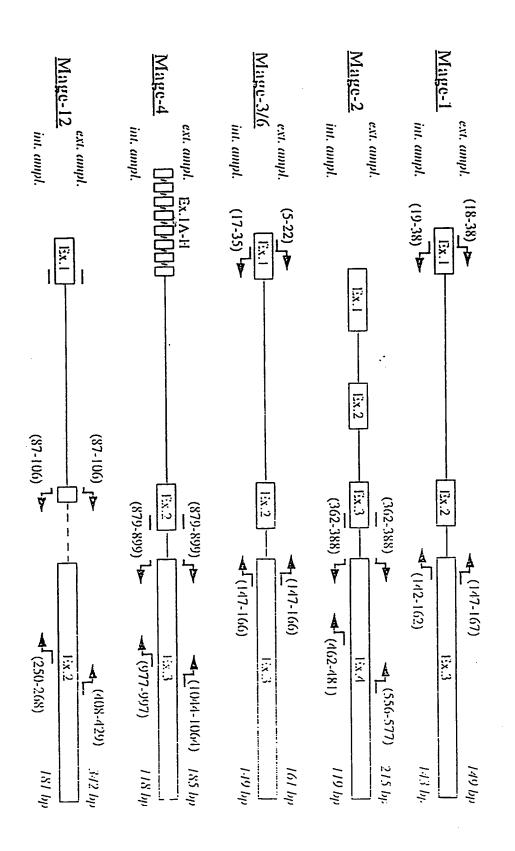


Fig.2: Number of BM aspirates from lung carcinoma patients with positive results in PCR-assay for at least one MAGE and in CK-Immunocytochemistry:

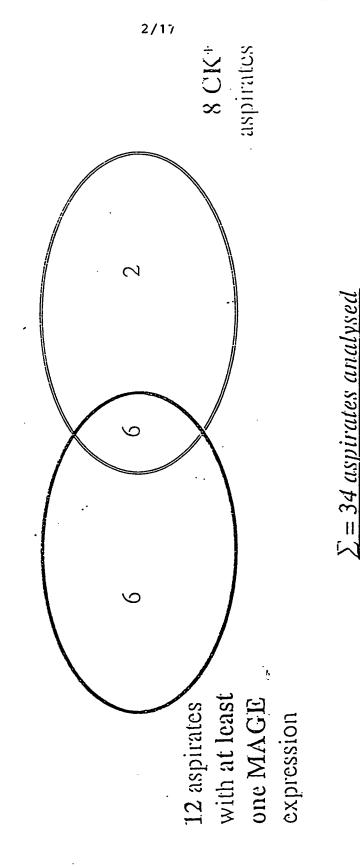


Fig. 3:. Number of prostate carcinoma patients with positive results in PCR-assay for at least one MAGE and PSA, and in CK-Immunocytochemistry in BM of at least one aspiration site.

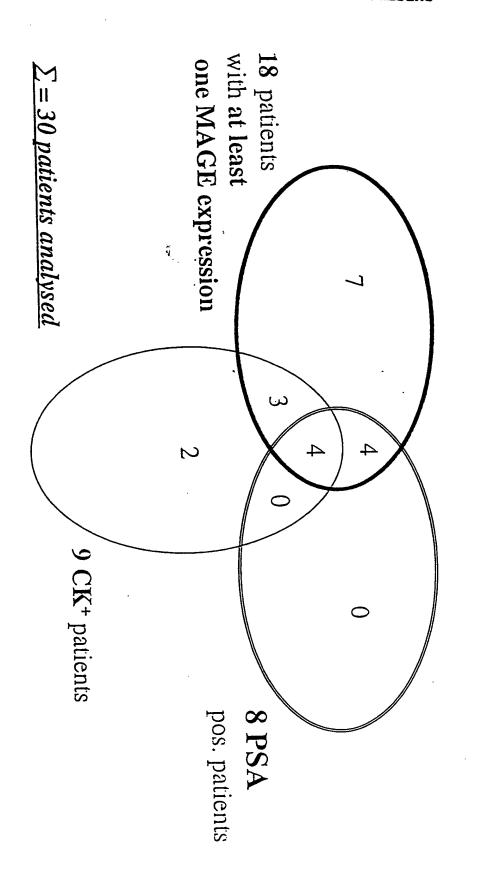
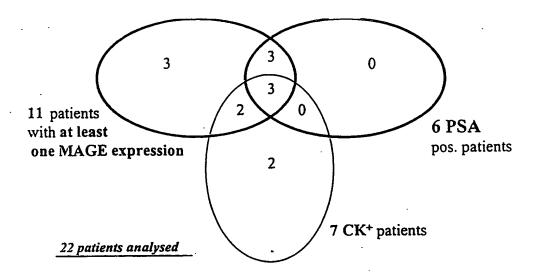
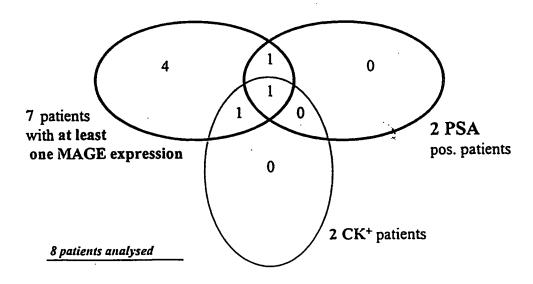


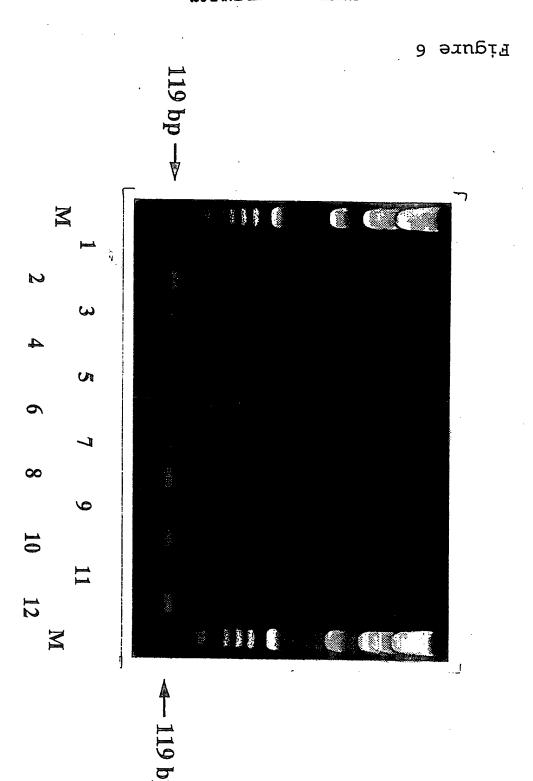
Fig. 4:. Number of prostate carcinoma patients with positive results in PCR-assay for at least one MAGE and PSA, and in CK-Immunocytochemistry in BM of at least one aspiration site

### a) analysis related to primary tumor

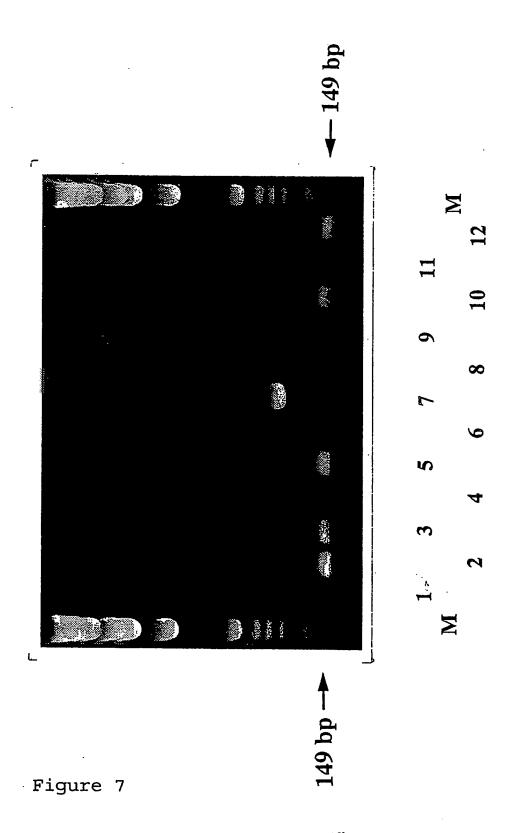


### b) analysis related to progressive disease



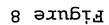


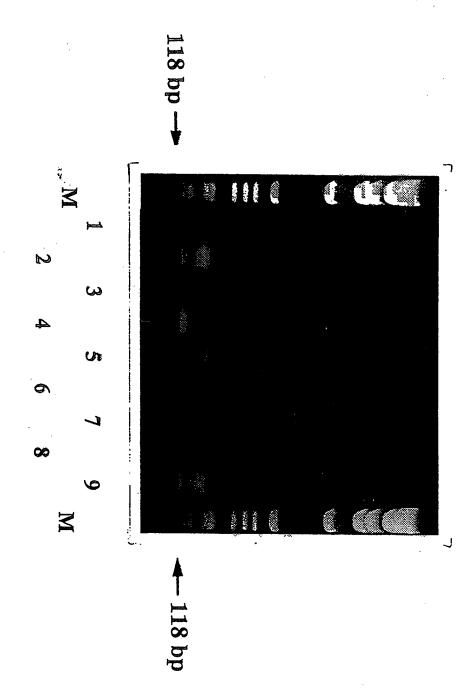
LT/9



SUBSTITUTE SHEET (RULE 26)

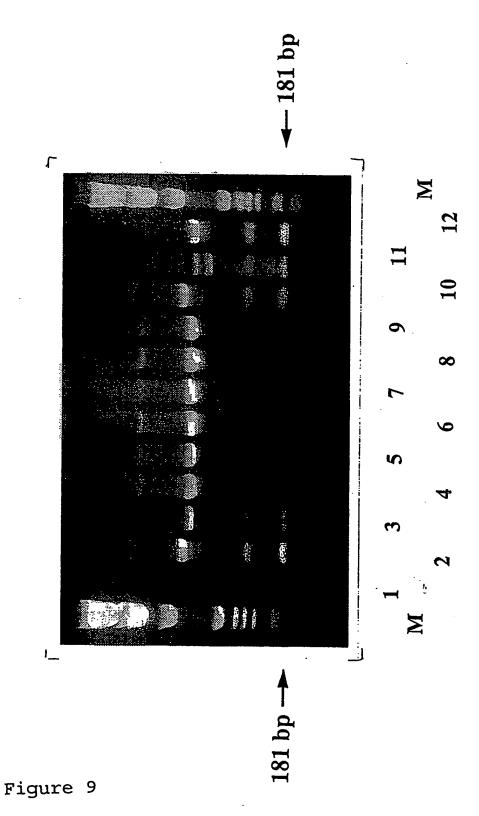
# SUBSTITUTE SHEET (RULE 26)



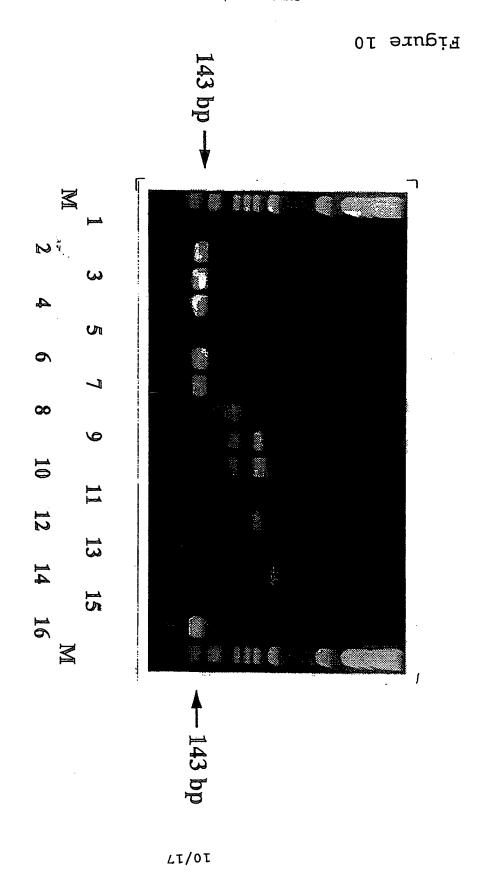


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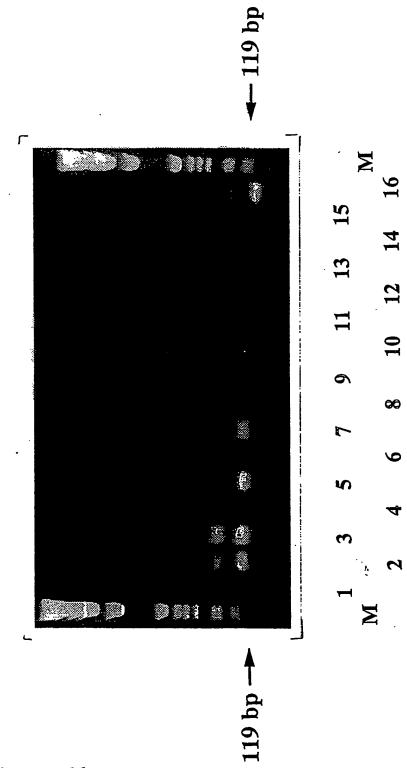
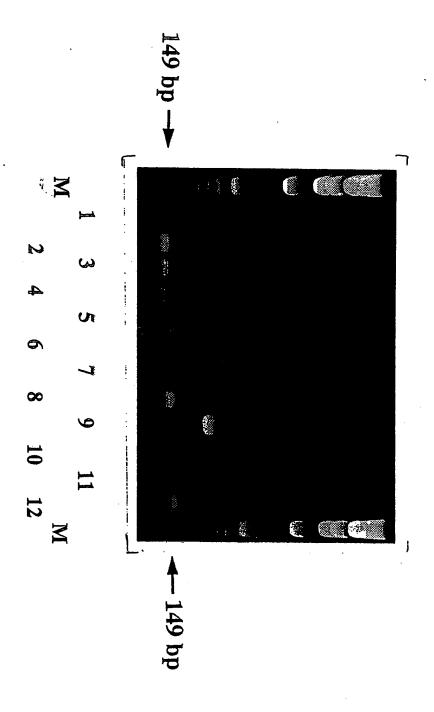


Figure 11

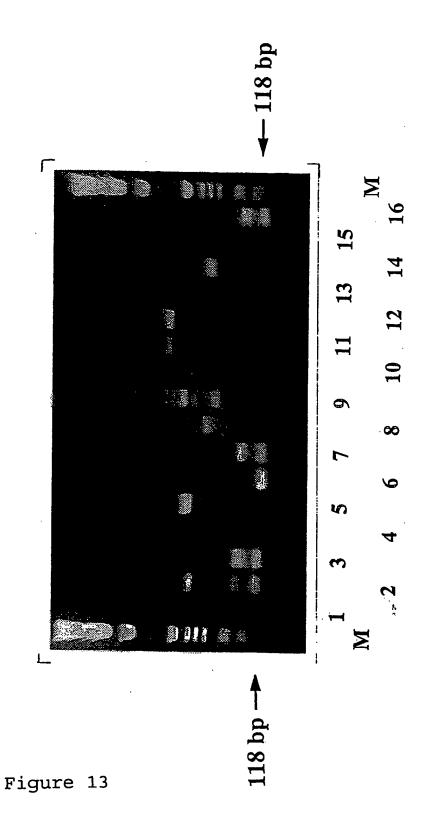
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Figure 12



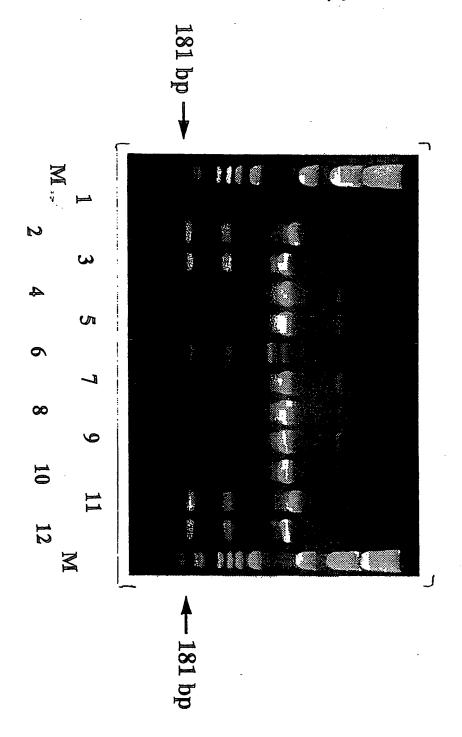
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Figure 14



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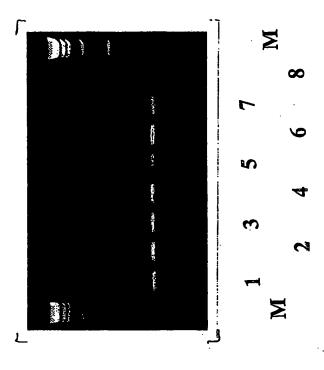
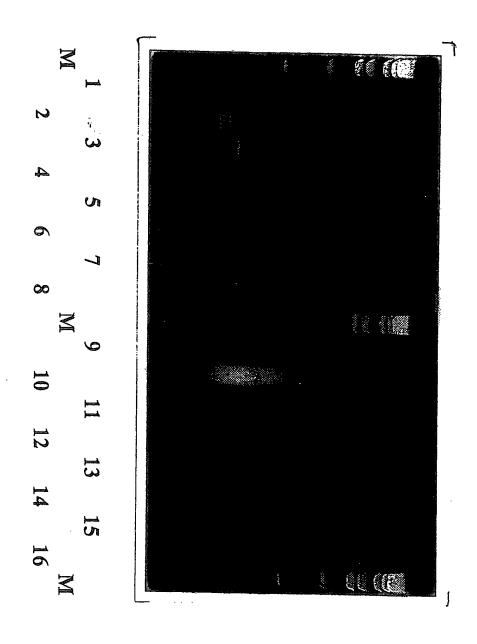


Figure 15a

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## Figure 15b



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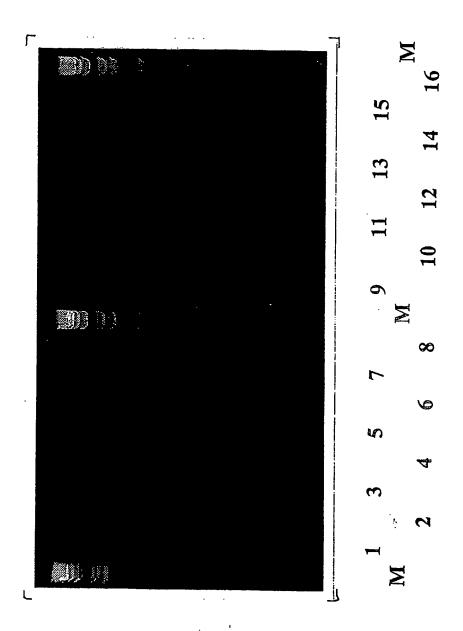


Figure 15c

Knehr, M NL - 2280 HV Rijawik Tol. (+31-70) 340-2040, Tx. 31 651 epo ni, Fax: (+31-70) 340-3016 European Petent Office, P.B. 5818 Petentlean 2 Authorizad officer A21 out to seerbbs gnillam bits email 12/10/1998 30 September 1998 Date of mailing of the international search report Cate of the actual completion of theinternational search g, qocriment member of the same patent family "P" document published prior to the international filling data but international leads to the prior prior to the calmed "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive arep when the ments, such combination being obvious to a person slittled in the art. "O" document referring to an oral disclosure, use, exhibition or T. querment which may throw doubts on priority distints) or which which may throw doubts on priority or shortlers. "X" document of particular nelevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone E ashler document but published on or after the international "A" document defining the general state of the art which is not considered to be of particular relevance "T" later document published after the interhational filling date or priority date and not in conflict with the application but clied to understand the principle or theory underlying the Special categories of cited documents: xerms ni beteli ena erechnem ylimst ineta? Further documents are listed in the continuation of box C. --/and the claims \* \* see in particular page 32, line 23 see the whole document cited in the application CENELIC2 INZL (N2)) SQ 26bfcmpcr 1360 1-13 MO 96 29430 A (WAYNE JOHN CANCER INST ; NAT X Relevant to claim No. Citation of document, with indication, where appropriate, of the relevant passages C. DOCUMENTS CONSIDERED TO BE RELEVANT Electronic data base consulted during the international search (name of data base and, where practical, search terms used) Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched CISO COIN 9 J4I Minimum documentation searched (classification system followed by classification symbols) 8. FIELDS SEARCHED According to international Patent Classification (IPC) or to both national classification and IPC A CLASSIFICATION OF SUBJECT MATTER

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